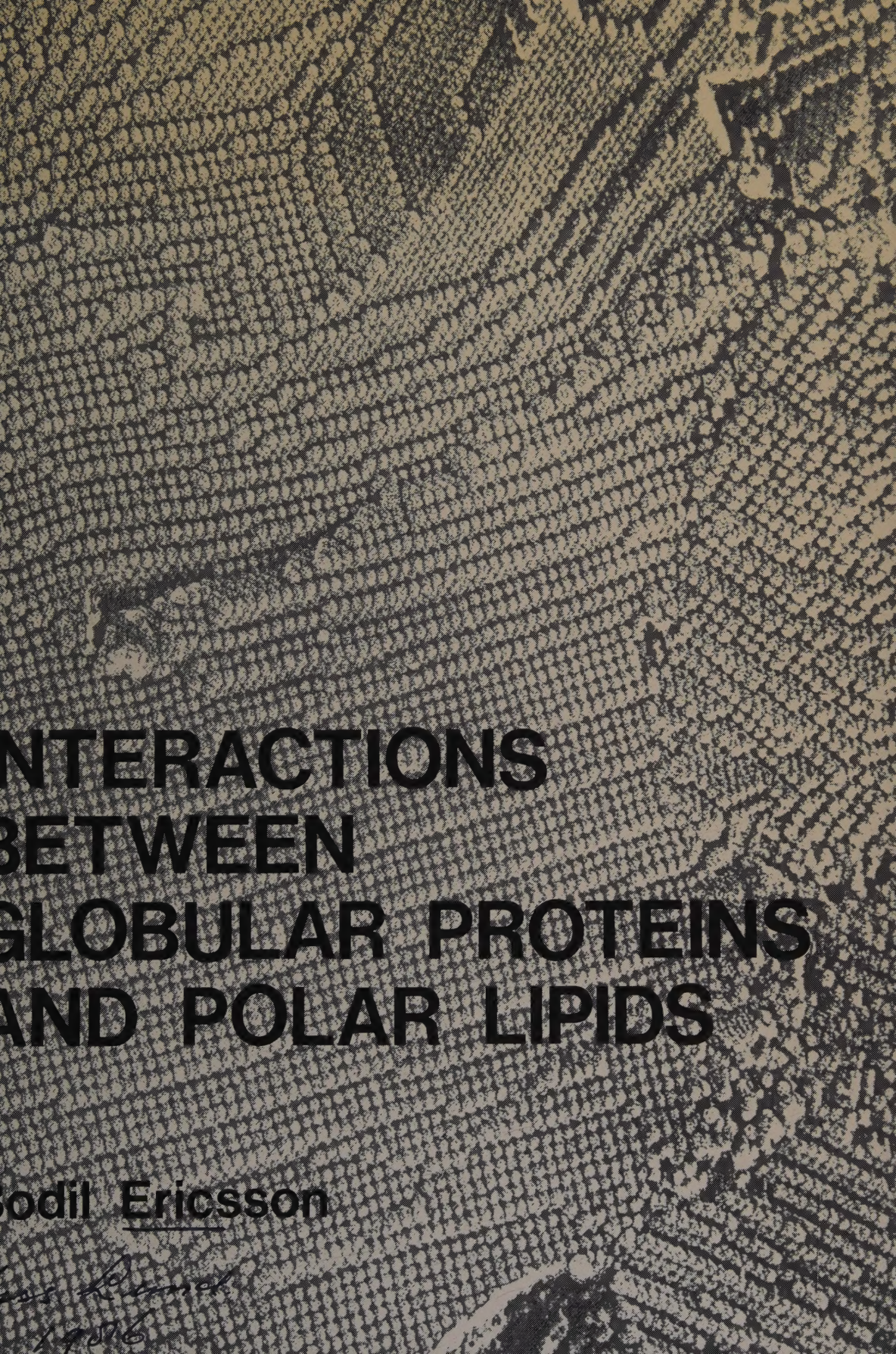


The background of the entire cover is a high-magnification electron micrograph showing the characteristic hexagonal or honeycomb-like pattern of a lipid bilayer. The pattern consists of dark, interconnected lines forming a mesh of small, irregular polygons, representing the hydrophobic tails of the lipids. The overall color is a monochromatic, grainy brown and black.

INTERACTIONS BETWEEN GLOBULAR PROTEINS AND POLAR LIPIDS

Udöil Ericsson

Udöil Ericsson
1986

INTERACTIONS BETWEEN GLOBULAR PROTEINS AND POLAR LIPIDS

BY

BODIL ERICSSON



LUND 1986

INTERACTION BETWEEN MONOLYMER AND WATER



The cover shows an electron micrograph of a monoglyceride-cytochrome c-water cubic phase.

(By Tadeusz Gulic-Krzywicki, Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette, France).

PUBLICATIONS

This thesis is based on the following papers, which will be referred to by Roman numerals in the text:

- I A Cubic Structure Consisting of a Lipid Bilayer Forming an Infinite Periodic Minimum Surface of the Gyroid Type in the Glycerolmonooleat-Water System.
S.T. Hyde, S. Andersson, B. Ericsson and K. Larsson
Z. Kristallogr. 168, 213-219 (1984)
- II A Cubic Protein-Monoolein-Water Phase.
B. Ericsson, K. Larsson and K. Fontell
Biochim. Biophys. Acta 729, 23-27 (1983)
- III Protection of Ovalbumin against Irreversible Heat Denaturation by a Cationic Amphiphile at High Concentration.
B. Ericsson, P.-O. Hegg and K. Mårtensson
J. Fd Technol. 18, 11-19 (1983)
- IV Effect of Cationic Amphiphiles and Temperature on Lysozyme Conformation.
B. Ericsson and P.-O. Hegg
Manuscript
- V Surface Behaviour of Adsorbed Films from Protein-Amphiphile Mixtures.
B. Ericsson and P.-O. Hegg
Progr. Colloid & Polymer Sci. 70, 92-95 (1985)
- VI Effects of Amphiphiles on Trypsin Activity and Conformation.
B. Ericsson, P.-O. Hegg and K. Mårtensson
Submitted to J. Dispersion Science Technology

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1. INTRODUCTION

Proteins and polar lipids are major building units in biological systems. They have a very important physical property in common - an inherent amphiphilic nature - which provides the driving force for formation of association structures of lipids, as well as for the folding of a polypeptide chain to form the unique conformation of a native protein. This dualistic character is also of great importance for the formation of composite structures, such as the cell membranes and the blood lipoproteins.

Interactions between proteins and lipids have received intensive interest for several decades, and a vast number of reports have occurred. The main concern has been to gain a better understanding of biological membranes (cf. Tanford, 1980; Yeagle, 1984). Water-soluble polar lipids are used for membrane solubilization (cf. Helenius and Simons, 1975), but also for characterization of membrane associated proteins (outlined by Tanford and Reynolds, 1976). In many model studies on the binding of lipids to proteins, water-soluble compounds have been used (cf. reviews by Steinhardt and Reynolds, 1969; Steinhardt, 1975; Makino, 1979). The unfolding of water-soluble proteins by synthetic polar lipids has contributed to the knowledge of protein structure, properties and function (cf. review by Lapanje, 1978). A well-known application originating from the investigations on unfolding is the sodium dodecylsulphate polyacrylamide gel electrophoretic (SDS-PAGE) determination of protein molecular weight (Shapiro et al., 1967; Weber and Osborn, 1969). The fundamentals of SDS-PAGE still receive much interest (cf. Hamed et al., 1983).

Proteins and lipids as well as their mutual interaction contribute significantly to the physical properties of many systems of technological interest. Protein structure, and thereby function and properties, are affected in various ways by polar lipids (cf. Brown, 1984). The intention with the investigations summarized in this thesis has been to gain a better understanding of protein-lipid interactions in aqueous medium and to elucidate the technological relevance of such interactions at conditions relevant for food production.

Protein-lipid interactions were investigated over a broad concentration range. In dilute solution lipids have been modelled by water-soluble, mostly synthetic polar lipids, while long-chain monoglycerides have been employed for the studies in condensed systems (presented in Table 1).

Water-soluble globular proteins have been used throughout these studies and in Table 2 are given some characteristics of the main proteins chosen.

The polar lipids employed as model substances are also interesting per se. Monoglycerides are frequently used as food additives, and they are also a main product in intestinal hydrolysis of dietary fats. Knowledge of the phase behaviour, as well as the interaction with water-soluble macromolecules, is therefore of fundamental interest.

In every-day life we are exposed to water-soluble synthetic polar lipids. Long-chain quaternary ammonium compounds are found in for example products for personal care and in pharmaceutical products, as well as in rinses and disinfectants for household and industrial use, for instance in the food industry. Hexadecylpyridinium chloride (see Table 2) e.g. is a very effective and commonly used bactericide. Recently, Ashman et al. (1986) have shown these compounds to be highly immunosuppressive - even more potent than the dominating immunosuppressive drug used today, cyclosporin A. This makes the interaction of cationic surfactants with biological macromolecules of special interest.

Protein-polar lipid interactions were studied by various physico-chemical methods. At room temperature the main methods used were low-angle X-ray diffraction, circular dichroism and the drop-volume method for surface tension measurements. Differential scanning calorimetry and specific optical rotation were used at increased temperatures.

TABLE 1

Selected properties of the proteins mainly used as model compounds.

Protein	MW	pI	S-S bridges	α -helix (%)	Molecular dimension (Å)	Action
Lysozyme (hen eggwhite)	14 500 ¹	11.0-11.3 ¹	4 ¹	45 ³	45x30x30 ¹ prolate ellipsoid	enzyme with activity against the mucopolysaccharide cell walls of bacteria ¹
Ovalbumin (hen eggwhite)	45 000 ⁴	4.5-4.9 ²	1 ⁵	30 ⁶	-	storage protein
Serum albumin (bovine)	66 700 ⁷	4.7-4.9 ⁸	17 ⁷	50 ⁷	140x40x40 ⁷ prolate ellipsoid	transport of free fatty acids in the circulatory system of mammalia ⁷
Trypsin (bovine pancreas)	24 000 ⁹	10.5-10.8 ⁹	6 ⁹	14 ¹⁰ (at pH 9)	-	proteolytic enzyme ⁹

¹Imoto et al. (1972)²Rhodes et al. (1958)³Levitt and Greer (1977)⁴Cannan et al. (1941)⁵Fothergill and Fothergill (1970)⁶Ying-Yet and Jirgensons (1977)⁷Peters Jr. (1985)⁸Eigel et al. (1984)⁹Keil (1971)¹⁰Lazdunski and Delage (1965)

TABLE 2

Selected properties of the polar lipids mainly used as model compounds.

Polar lipid (abbreviation)	Structural formula	cmc (25°C, electrolyte free aqueous medium)
Hexadecylpyridinium chloride (HPC, CPC)	 $\text{N}^+-\text{CH}_2(\text{CH}_2)_{14}\text{CH}_3$	0.9 mM
Hexadecyl lysine ester dihydrochloride (HLDC)	$\text{H}_3\text{N}^+-(\text{CH}_2)_4-\overset{\text{O}}{\underset{\text{NH}_3^+}{\text{C}}}-\text{O}-\text{CH}_2(\text{CH}_2)_{14}\text{CH}_3$	- (low solubility, forms liposomes in diluted aqueous medium)
Dodecylammonium chloride (DAC)	$\text{H}_3\text{N}^+-\text{CH}_2(\text{CH}_2)_{10}\text{CH}_3$	14 mM ¹
Sodiumdodecyl sulphate (SDS)	$^-\text{O}_3\text{S}-\text{O}-\text{CH}_2(\text{CH}_2)_{10}\text{CH}_3$	8.3 mM ¹
1-Monocaproin ¹	$\text{H}_2\text{C}-\overset{\text{O}}{\underset{\text{HO}-\text{CH}}{\text{C}}}(\text{CH}_2)_4\text{CH}_3$ CH_2OH	160 mM
1-Monolein	$\text{H}_2\text{C}-\overset{\text{O}}{\underset{\text{HOCH}}{\text{C}}}(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7\text{CH}_3$ CH_2OH	- (low solubility, forms liquid crystalline phases)

¹Data taken from Murkerje and Mysels (1971).

2. BEHAVIOUR OF THE PURE COMPONENTS

The origin of the physical behaviour of amphiphilic substances are found in the ability of the hydrophobic moieties of the molecule to diminish the area in contact with water, balanced by the strong tendency for hydration of the polar groups.

2.1 Globular Proteins

The architecture of the three-dimensional structure of proteins (the conformation) has a hierarchical nature. The units of the polypeptide chain, the amino acids, are of two main types: nonpolar, which provide a strong driving force for folding, and polar (uncharged and charged) with high affinity for aqueous solvent. The sequence of the polypeptide chain (the primary structure) governs the folding into structural units (the secondary structure). The association of structural units into domains, tertiary and quaternary structures, gives each protein the unique conformation connected with its action and specificity.

2.1.1 The Secondary Structure

The secondary structure units are either repetitive or nonrepetitive. The repetitive forms, stabilized mainly by intra-chain hydrogen bonds, are of α -helical, of parallel or antiparallel β -sheet types. In water-soluble proteins, the α -helical and antiparallel β -sheet foldings are most commonly located at the surface of the protein. This is assigned to the tendency for a certain degree of periodicity of hydrophobicity - hydrophilicity in the amino acid sequence in the polypeptide chain involved, giving a two-faced image of those foldings. The parallel β -sheet is composed mainly of non-polar amino acids and is thus always found in the interior of the folded protein (Richardson, 1981). This is the least stable of the repetitive foldings. Membrane proteins, which are mainly lipophilic, exhibit α -helix and β -sheet formations in the hydrophobic region, located in the lipid bilayer (cf. Unwin and Henderson, 1984).

A nonrepetitive, but constrained structure of great importance for protein folding and stability is the tight turn types (Veukatachalam, 1968), also called β -turns or hairpin bends. The turns, connecting different stretches of secondary structure, have been suggested to provide the direction in the folding process of the native conformation (Rose et al., 1976). The turns occur typically at the surface of the protein, and are often stabilized by hydrogen bonding with the surrounding water (Franks, 1985).

Additional structures, referred to as "other" or as random-coil, are often as highly organized and constrained as the regular ones, but more difficult to describe due to their non-repetitive nature (Richardson, 1981). These are often stabilized by specific side-chain interactions. Disordered regions are also found, sometimes associated with the specific action of the protein. The binding of ligands, substrates etc. to such regions might introduce order (cf. trypsin in paragraph 4.3).

Many proteins have been found to fold in domains (cf. Wetlaufer, 1981). Lysozyme has been shown to be composed of two domains (Yonath et al., 1977) and bovine serum albumin of three domains (Teale and Benjamin, 1976) which fold, and unfold, independent of each other. The building up of proteins into domains is thought to reflect different evolutionary developments. The domains might also facilitate rapid self-assembly of large proteins (Wetlaufer, 1973).

2.1.2 The Tertiary Structure

The tertiary (supersecondary) structure of globular proteins in solution is mainly stabilized by interaction with the solvent. The different folding units either require shielding against solvent contact and are thus found in the core of the proteins, or are more polar on one side, and thus stabilized at the surface of the protein. Further, disulfide bridges provide specificity and stability in the joining of the secondary structure elements.

Polar interaction, both interior and exterior, are of dominating importance for protein stability. The contribution from exterior charged residues to the stability arises not only from the strong hydration tendency, but also from formation of salt bridges on the protein surface (Perutz, 1978). The stability of proteins is at maximum at the isoelectric point and decreases on either side due to the expansion of the polypeptide chain caused by increased charged density. Also in intermolecular protein interactions, as aggregation and gelation, electrostatic interaction is of utmost importance. The DLVO-theory, combining the long-range London-van der Waals forces with the repulsive force deriving from the electrical double layer surrounding charged "surfaces", has been applied to explain colloidal stability (cf. Mulder and Walstra, 1974; Walstra, 1983).

2.1.3 Solvent interactions

The prevalent picture of native globular proteins as consisting of a hydrophobic core covered by a hydrophilic shell is an oversimplification. Although water soluble, native globular proteins have a not negligible amount of non-polar groups situated at the surface accessible to the solvent. In lysozyme for example, about 17% of the nonpolar groups project into the solvent and, in addition, 50% more are at least partially exposed to the solvent (Browne et al., 1969; Shrake and Rupley, 1973).

The amphiphilic character of globular proteins provides an explanation for many intermolecular processes. The insertion of nonpolar groups in aqueous solution induces entropically unfavourable reorientation of the water molecules. A process that will relieve some of the constrain in this water will be thermodynamically favoured, and this is the basis of the hydrophobic interaction (Tanford, 1980). The hydrophobic force has been measured by Israelachvili and Pashley (1982), who found that the attractive long-range force between hydrophobic surfaces in aqueous solution is an order of magnitude stronger than the van der Waals attraction forces, and decays exponentially with distance in the range 0-10 nm.

The intermolecular attraction by the hydrophobic force and the van der Waals force are balanced by an electrostatic repulsion force and by the hydration shells surrounding the protein molecules. Not only the primary hydration layer, associated by hydrogen bonds to polar surface groups, but also water molecules further out from the protein are involved. Here the water-water interactions are strongly influenced by the surface structure of the protein (Franks, 1977). The occurrence of repulsive forces at short distances between neutral lipid bilayers in water was discovered by Rand, Parsegian and co-workers in 1976, and they introduced the concept "hydration forces" (LeNeveu et al., 1976, 1977; Rand, 1981). Accurate measurements of these forces between mica surfaces (also mica surfaces covered by amphiphile bilayers) have been reported by Israelachvili and Pashley (Israelachvili and Pashley, 1983; Pashley and Israelachvili, 1981). At these conditions, oscillations between repulsive and attractive forces, depending upon water layer thickness, were observed. The origin of the hydration forces is the energy needed to dehydrate approaching surfaces containing ionic or polar species. The significance of the hydration force in technologically important systems has been discussed by Parsegian (1982). The suggestion that the hydration force also is of importance for

the specific assembly of subunits to form the quaternary structure of proteins (Parsegian, 1982; Rau et al., 1984) indicate the importance of this particular force for intra- and intermolecular protein stability.

2.1.4 Denaturation

Denaturation (or unfolding) of a globular protein denotes "a process in which the conformation of the polypeptide chain within the molecule is changed from that typical of a native protein to a more disordered arrangement" (Kauzmann, 1959). This definition of the denaturation process also includes changes in conformation accompanying limited hydrolytic cleavage of peptide linkages.

The native state, being a set of states in rapid equilibrium with each other, distributed around an average conformation, is only marginally stable. The free energy of unfolding corresponds only to the breaking of 2 to 5 hydrogen bonds per molecule (10 to $20 \text{ kcal} \cdot \text{mol}^{-1}$) (Finney, 1982), which implies the importance of the surrounding medium (water) for the stability of the native conformation. Denaturation can thus be achieved by denaturants, which disturb the protein-solvent interaction (e.g. urea, guanidinium hydrochloride, organic solvents, sodium dodecylsulphate, acids, alkali). Also physical changes (temperature or pressure) can act as denaturants. The unfolding process normally occurs within a narrow concentration range of denaturant. In spite of this apparent two-state process of protein unfolding, studies using different techniques have shown the existence of intermediate states (cf. Pace, 1975).

The structure of the denatured state depends on the denaturant, and is often poorly characterized. It is generally assumed that protein denatured by acid or heat retain a considerable amount of ordered structure, while urea and guanidinium hydrochloride at high concentrations bring about a more disordered state (Tanford, 1968). Pfeil and Privalov (1976) have, however, shown that the thermodynamic states of lysozyme denatured by heat and by guanidinium hydrochloride are indistinguishable.

Denaturation of proteins by organic solvents results in a structure with large fractions of the polypeptide chain in α -helical conformation (Tanford, 1980). This structure is thought to consist of helical segments with flexible joints, and with most of the hydrophobic side-chains exposed to the solvent. Also denaturation by long-chain alkyl sulphates, like SDS,

brings about an increase in α -helical content for certain proteins (Jirgensens, 1976; Mattice et al., 1976). A simplified rule seems to be that SDS increases the α -helical content in proteins with low and intermediate amounts of α -helix in native state (e.g. concanavalin A, β -lactoglobulin and ovalbumin). The inverse appears true for proteins with a high amount of α -helix in the native state (e.g. myoglobin and serum albumin). Protein molecules with exposed hydrophobic groups are likely to bind hydrophobic ligands. The successive binding of SDS creates new hydrophobic binding sites, and the association stabilizes α -helical folding at the expense of nonrepetitive structure. The free energy gained by this process might by far exceed the unfavourable free energy change of unfolding the native conformation (cf. Tanford, 1980). At high amounts of SDS the protein-SDS complex is considered to consist of a polypeptide chain with high flexibility, to which SDS molecules bind to form micelle-like clusters (cf. Mattice, 1976).

The amphiphilic nature of proteins cause them to adsorb and orient at polar-nonpolar interfaces. When adsorbed from aqueous solution at a hydrophobic interface, the protein molecule loses its native conformation (MacRitchie, 1978). In the case of a flexible polymer, the adsorption results in a structure comprised of three parts:

- 1) the trains, which are segments adsorbed at the interface,
- 2) the loops, which are segments of the chain penetrating the bulk phases,
- 3) the tails i.e. the ends of the polymer chain.

Adsorption of proteins at low interfacial pressure (low interface and bulk concentration) results in a high amount of train conformation, while at increased surface pressure the amount of segments in loop conformation increases (MacRitchie, 1978). For globular proteins, there is reason to believe that segments of secondary structure are retained at the interface (MacRitchie, 1978). Graham and Phillips (1979) have shown that adsorption of globular proteins at the air-water interface not results in a fully extended polypeptide chain in train configuration at any surface concentration. The remaining ordered structure presumably includes disulphide bridges, stabilizing the loops, and α -helical segments, which are known to be stabilized at interfaces (cf. section 2.1.1). The degree of unfolding of globular proteins at liquid interfaces seems to depend amongst others on the surface pressure, i.e. at increased surface pressure the protein mole-

cules at the interface attain a more compact structure, which, however, deviates from the native conformation (MacRitchie, 1978).

2.2 Polar Lipids

The common feature for self-association of polar lipids in an aqueous environment is the liquid like nature of the hydrocarbon chains and the formation of a polar interface, separating the hydrocarbon and water regions. Polar lipids can be divided into two classes on the basis of their interaction with water:

1. Lipids, which are water-soluble in monomeric and micellar form,
2. Lipids, with very low water solubility, but with the ability to swell into liquid crystalline phases.

2.2.1 Micellar Phases

The water soluble polar lipids (e.g. ionized fatty acids, bile salts and synthetic surfactants) have monomeric solubility in the mM range, and form micelles at higher concentrations (Fig. 1). The critical micelle concentration, cmc, is considered to be a narrow concentration range, within which aggregates start to form by a strong co-operative process (Lindman and Wennerström, 1980). The driving force for micellization is the hydrophobic force. Cmc for single chain amphiphiles decreases with increasing chain length, and for ionic amphiphiles cmc also depends on ionic strength, as addition of salt reduces the electrostatic repulsion between the charged headgroups. Increased temperature has only a moderate influence on cmc, once the temperature has exceeded the critical temperature (Krafft temperature) (Lindman and Wennerström, 1980). The size and shape of the micelles formed (spherical, globular or cylindrical) depend both on the geometry of the molecule and on the total concentration of the amphiphile. The common visualization of micelles as being totally covered by polar groups is misleading. For space reasons, the polar groups are only partly able to cover the surface of the micelle (cf. Fontell, 1981).

The micellar structure in water-poor solution is of the inverse type (see Fig. 1), i.e. water is included in a lipid matrix. This kind of structure, denoted L2-phase according to the nomenclature of Ekwall (1975), is formed at low water content. When oil is solubilized into this lipid matrix the structure is also often referred to as a "microemulsion". The structure

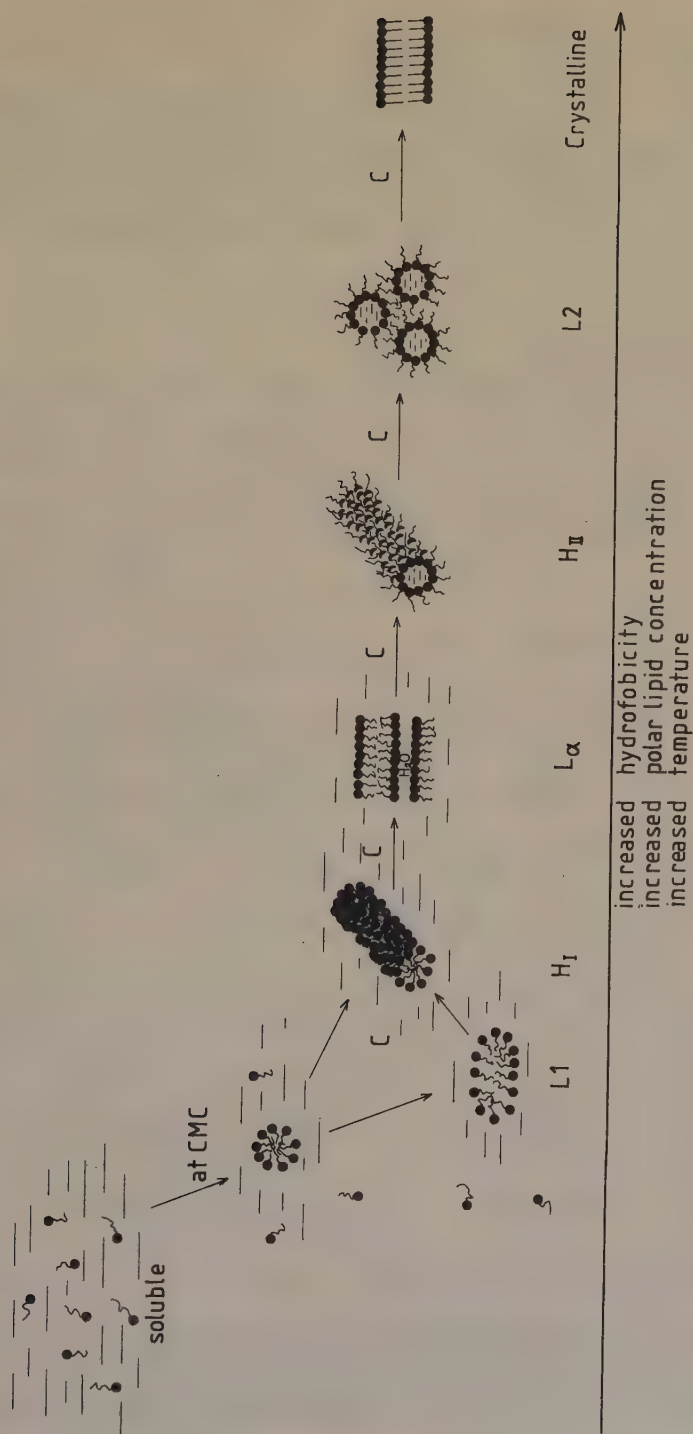


Figure 1. Commonly formed association structures by polar lipids. Phase transitions can be induced by changes in water content or temperature. With decreasing water content or increasing temperature, class 1 lipids usually show the transitions: $L1 \rightarrow H_I \rightarrow L_\alpha \rightarrow L2$, whereas lipids belonging to class 2 follow the sequence: $L_\alpha \rightarrow H_{II} \rightarrow L2$.

of the L2-solution proposed in the monoglyceride-water systems consists of finite water-units separated by a continuous bilayer system (Larsson, 1979; Gulik-Krzywicki and Larsson, 1984). The short-chain monoglyceride, 1-monocaproin (V) exhibits a unique phase behaviour. It is the sole binary lipid-water system known, which forms only one liquid phase at all composition above 0°C (Larsson, 1967). At compositions above approximately 50% water, the micelles formed are of normal L1-type, with cmc in distilled water at 160 mM (V), and at lower water content the micelles are of the L2-type.

2.2.2 Liquid Crystalline Phases

A common feature of the two classes of polar lipids discussed above is the tendency to form lyotropic liquid crystalline phases. As indicated in Figure 1, several different liquid crystalline phases can occur. With decreasing water content, polar lipids often exhibit the sequence: hexagonal phase (H_I) $\rightarrow$ lamellar phase (L_α) (class 1 lipids) and $L_\alpha \rightarrow$ reversed hexagonal phase (H_{II}) (class 2 lipids). Cubic liquid-crystalline phases occur often between these. Phase transitions can also occur with changes in temperature; with increasing temperature the sequence of thermal phase transitions is usually the same as that given above (Fig. 1). The formation of a particular phase is determined by several factors, one of which is the geometry of the amphiphilic molecule and the packing restrictions for the different lipid phases (Israelachvili et al., 1976). The geometry of a polar lipid can be described by the ratio $v(a \cdot l)^{-1}$, where v is the volume of the hydrophobic moiety, l is the effective hydrocarbon chain length, and a is the mean area occupied by the amphiphile at the polar-nonpolar interface. If the hydrocarbon chain is fluid, the self-assembly in dilute systems can be predicted by $v(a \cdot l)^{-1}$ according to Figure 2 (Israelachvili et al., 1976, 1980). Generally speaking, a low volume-to-area ratio facilitates the formation of structures of the "oil-in-water" type ($L1$, H_I), while a high ratio gives rise to aggregates of the inverse type ("water-in-oil"; H_{II} , $L2$). Conditions changing the geometrical ratio for an amphiphilic molecule will ultimately give rise to phase transitions. Increased temperature e.g. will increase chain mobility and thereby increase the volume-to-area ratio, explaining the often seen thermally induced transition $L_\alpha \rightarrow H_{II}$. Decreased hydration will decrease the head group repulsion, resulting in a decreased interface area and thus in an increased volume-to-area ratio.

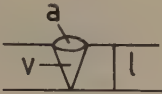
POLAR LIPID	MOLECULAR GEOMETRY	FORMED STRUCTURE
CLASS 1 LIPIDS Single-chain lipids with a large polar group e.g. SDS, DAC, HPC, lysolecithin	 $\frac{v}{a \cdot l} < \frac{1}{3}$  $\frac{1}{3} < \frac{v}{a \cdot l} < \frac{1}{2}$	SPHERICAL AGGREGATES (L1)  CYLINDRICAL AGGREGATES (L1, H_I) 
CLASS 2 LIPIDS Single-chain lipids with a small polar group Double-chain lipids with a large polar group e.g. monoglycerides, lecithin, sphingomyelin, diglycodiglycerides, phosphatidylethanolamine	 $\frac{v}{a \cdot l} \sim 1$	CURVED BILAYERS (liposomes, C)  PLANAR BILAYERS (L_α) 
CLASS 2 LIPIDS Double-chain lipids with a large nonpolar group, unsaturated chains e.g. monoglycodiglycerides, mixtures of monoglycerides-diglycerides	 $\frac{v}{a \cdot l} > 1$	INVERTED AGGREGATES (L2, H_{II}) 

Figure 2. Relationship between the geometry of amphiphilic molecules and the structures formed in aqueous systems. The favoured structures are the result of the interplay between entropy, favouring small aggregates, and packing restrictions which strive for large aggregates.

(Adopted after Israelachvili et al., 1980).

The structures of the most commonly found mesophases are schematically shown in Figures 1 and 2. The main feature of the structure of these lipid phases was determined by Luzzati and co-workers in the early 60's by X-ray diffraction (reviewed by Luzzati, 1968). Later, spectroscopy studies have given considerable contributions to the understanding of the dynamic nature of these phases (cf. Davis, 1983). The lamellar type consists of stacked infinite lipid bilayers separated by water layers, while the hexagonal phases consist of infinite cylinders, having either a hydrocarbon core (H_I) or a water core (H_{II}). The existence of different cubic phases (C) and their possible locations are indicated in Figure 1. A general type of cubic structure consisting of rodlike aggregates has been stated by Luzzati et al. (1968). Another general type, based on lipid bilayers, has been analyzed in the present work, and will be discussed in detail below.

2.2.3 The Binary Aqueous Monoglyceride Systems

Phase diagrams of monoglyceride-water systems have been determined by Lutton (1965) and by Larsson (1967). Cubic phases are found in these systems when the acyl chain contains 14 or more carbon atoms. The cubic phase region is found on the water-rich side of the lamellar phase at isothermal conditions. In some systems, the cubic phase is transformed into the reversed hexagonal phase at increased temperature.

The phase diagram of 1-monoolein is shown in paper I (Fig. 2). This compound swell with water at room temperature to form the cubic phases in the water range 25-40% (w/w). The cubic structure occurring in this system was examined by X-ray diffraction combined with NMR diffusion measurements (Lindblom et al., 1979). A three-dimensional lipid-water bicontinuous structure was proposed, in which lipid bilayer hexagon units are arranged in a body-centered cubic lattice. This proposal was based on the relationship between the lamellar and the cubic lattice dimensions. It was later proposed that the structure was an infinite-periodic-minimal-surface (IPMS) (Larsson et al., 1980), where a lipid bilayer divides the aqueous medium into two equal water channel systems without any connection.

Recently Longley and McIntosh (1983) gave conclusive evidence for a cubic structure in the 1-monoolein-water system at the maximum swelling limit, with the lipid bilayer units forming a primitive cubic lattice. The structure of this cubic phase is shown in Figure 3. In the primitive lattice,

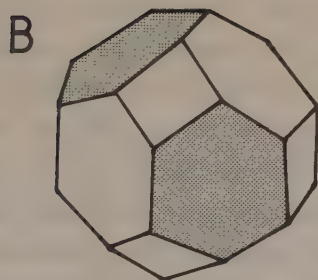
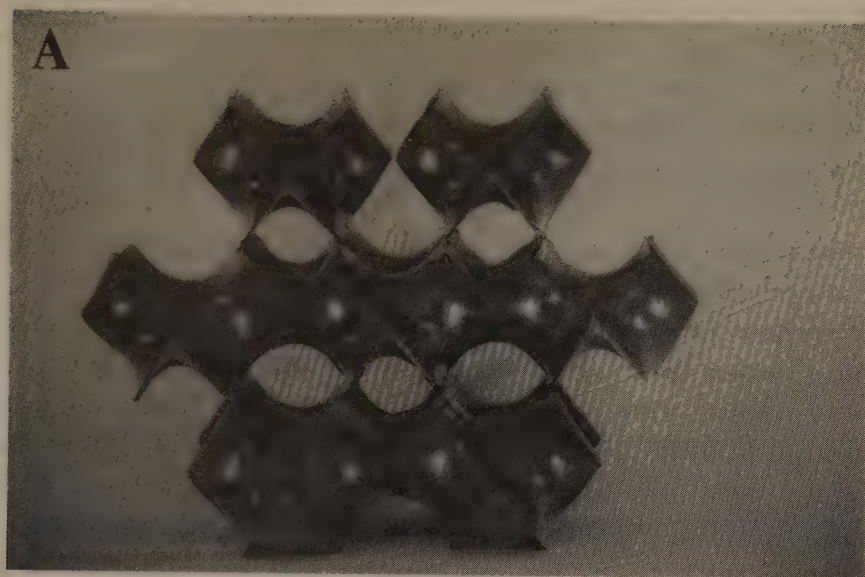


Figure 3. A. The IPMS of diamond type (D) corresponding to the primitive cubic phase.
B. The simplified structure unit of the D-phase. Four open hexagons form connections with joining neighbours.

four hexagons form connections with joining neighbours. The work of Longley and McIntosh (1983) revealed the remarkable occurrence of two cubic phases (Larsson, 1983) in the same region of the phase diagram. The earlier interpretation of only one cubic phase gave the wrong space group of the body-centered lattice discussed above. The true symmetry is $Ia\bar{3}d$, and the structure is defined by the gyroid surface as discussed below.

In (I) a model for the transition between the body-centered cubic phase and the primitive cubic phase in the 1-monoolein-water system is given. Both cubic structures have been proposed to be IPMS, which is the ideal way to separate a medium into two interpenetrating continuous systems of equal volume (cf. Shoen, 1970). The IPMS of these two cubic structures belong to different space groups. The primitive lattice corresponds to the diamond (D) type of IPMS, whereas the body-centered lattice corresponds to the gyroid (G) type. The IPMS is actually located in the gap between the methyl end groups of the bilayer, i.e. at the centre of the lipid bilayers. The G-surface is shown in Figure 1 in paper I with the two sides in different colours. Each side represents one of the two water channel systems.

Recently the mathematical relations between the three types of IPMS, the primitive type (P), the D- and the G-type, were revealed (Hyde and Andersson, 1985). Thus it was found that these surfaces are related by so-called Bonnet transformations, which means that (almost) energy-free transitions between for example the G- and D-surfaces can take place. The two cubic phases in the monoglyceride-water system was examined in this respect. It was shown that the lattice dimensions and the transition region between the two phases were in perfect agreement with the theory of Hyde and Andersson (1985), provided that the body-centered phase is the G-type and the primitive one is the D-type. On this basis it was proposed that the earlier suggested body-centered cubic P-type of structure does not occur, and that the structure instead is of the G-type. The fundamental features in the nature of the cubic phase stated by Lindblom et al. (1979), i.e. the three-dimensional bicontinuous lipid-water nature, is of course common to the cubic G- and D-phases.

The role of the "intercubic" phase transition for the formation of a homogeneous cubic phase containing water-soluble proteins will be discussed in paragraph 3.1.

The phase diagram of a sunflower oil monoglyceride mixture of food grade (Dimodan LS, kindly supplied by A/S Grinstedvaerket, Brabrand, Denmark) is seen in Figure 4.

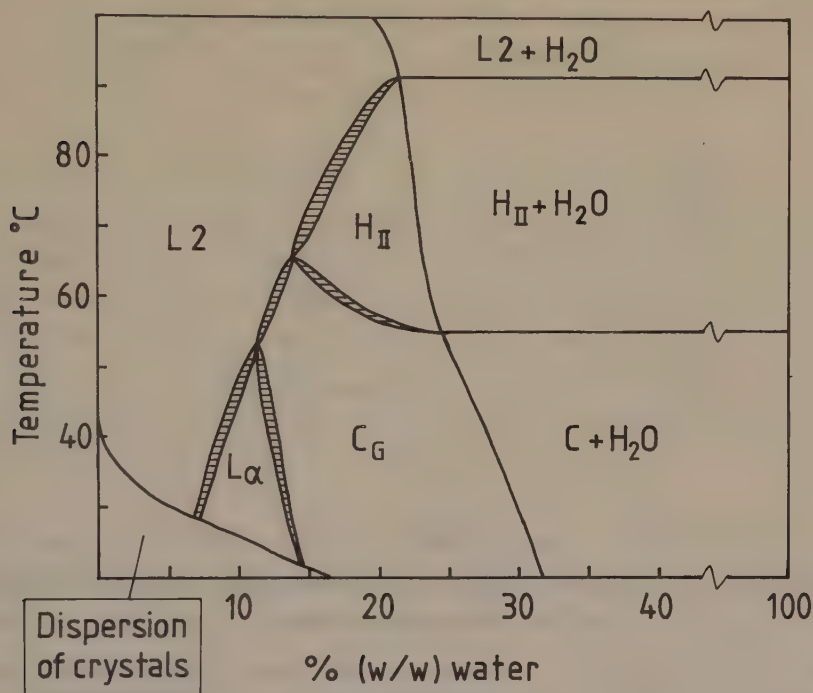


Figure 4. The phase diagram for the binary system sunflower oil monoglyceride/water.

The fatty acid composition of this mixture is given in Table 3. The region of the cubic phase is shifted towards lower water content, and the transition to the reversed hexagonal phase occurs at a lower temperature compared to the phase transitions in the 1-monoolein-water system. The X-ray diffraction pattern of samples at the maximum and minimum swelling limits are of the same character, indicating no difference in the cubic lattice. Thus the cubic $C_G \rightarrow C_D$ phase transition, which was seen in the 1-monoolein-water system, is not observed. As shown in Table 4, the X-ray spacings must be indexed according to the body-centered cubic lattice. It is not possible to fit the spacings into indices corresponding to a primitive lattice, since $(h^2 + k^2 + l^2) = 7$ cannot exist in cubic symmetry. It must be pointed out, however, that the phase diagram in Figure 4 does not show the same phase boundaries as an earlier reported phase diagram of a

TABLE 3

The fatty acid composition of the Dimodan LS preparation.

Fatty acid	%
C _{14:0}	0.1
C _{16:0}	7.1
C _{16:1}	0.1
C _{18:0}	4.8
C _{18:1}	21.8
C _{18:2}	63.9
C _{20:4}	1.4
C _{22:0}	0.7

TABLE 4

X-ray diffraction data of the cubic phase in the binary aqueous sunflower oil monoglyceride system.

Water % (w/w)	d(Å)	(h ² +k ² +l ²)	a ₀ (Å)	Water % (w/w)	d(Å)	(h ² +k ² +l ²)	a ₀ (Å)
18	45.7	6	112.0	> 32	52.6	6	128.8
	39.2	8	110.9		45.7	8	129.3
	30.1	14	112.4		34.3	14	128.3
	28.2	16	112.8		31.6	16	126.4
	25.2	20	112.7		28.7	20	128.4
	23.5	22	<u>110.2</u>		27.4	22	<u>128.5</u>
	Mean value		111.8		Mean value		128.3

different preparation of sunflower oil monoglycerides (Dimodan LS) (Larsson et al., 1980). For this preparation the maximum swelling limit of the cubic phase was reported to be around 50% (w/w) water at 20°C. Due to the phase boundary in the latter monoglyceride-water system, it seems plausible to assume that the $C_G \rightarrow C_D$ transition can take place in mixed monoglycerides at certain conditions. This is also confirmed by the protein incorporation properties of the cubic phases which is discussed below.

The difference noted between the two reported phase diagrams of sunflower oil monoglycerides, and the absence of the $C_G \rightarrow C_D$ transition in the former system might be due to the difference in the fatty acid pattern. A comparison between the fatty acid pattern for the monoglyceride mixture used here and the one used by Larsson et al. (1980) shows that the latter contained more $C_{18:1}$ (26.4%) and less $C_{18:2}$ (60.8%). Also variations in the (small) amounts of free fatty acids, di- and triglycerides in the monoglyceride preparations might influence the phase boundaries.

3. INTERACTIONS BETWEEN GLOBULAR PROTEINS AND LYOTROPIC MESOPHASES

Interactions between water-soluble globular proteins and liquid crystalline mesophases have been reported to depend on the possibility of electrostatic interactions between the protein and the polar groups of the lipid (Rand, 1975). Several investigators have reported interaction between anionic phospholipid bilayers (L_α -phase and liposomes) and monolayers with positively charged proteins, e.g. serum albumin and cytochrome c at acidic pH (Rand, 1971; Gulik-Krzywicki et al., 1969; De Kruijff and Cullis, 1980; Quinn and Dawson, 1969). The protein is found to be either associated to the polar interface of the mesophase by electrostatic interactions or to be incorporated in the lipid layers by a combination of hydrophobic and electrostatic attractive forces. Lipid phase transitions induced by protein have also been reported (cf. De Kruijff and Cullis, 1980). In most cases the reported interaction between water-soluble proteins and lipid mesophases lead to denaturation of the protein (cf. Rand, 1971).

3.1 Interaction Between Globular Proteins and Monoglyceride-water phases

The interaction between proteins, carrying different net charges, and monoglycerides, i.e. uncharged polar lipids, is reported in paper II. Thus the polar lipid-water-protein phases discussed in this thesis are of a different type from those reported earlier.

3.1.1 The Cubic Phases

The formation of composite cubic phases of globular proteins in native state and 1-monoolein is reported in paper II. The homogenous cubic samples containing protein showed the same characteristics regarding flow properties, microscopic appearance and X-ray diffraction as the cubic binary 1-monoolein-water phase examined earlier. The X-ray diffraction data were thus indexed according to the body-centered cubic lattice (II). Due to the evidence for two cubic phases (I), however, the X-ray diffraction data reported in II have been reconsidered and an alternative indexing is given in Table 5. X-ray diffraction data of two additional samples are included. The X-ray data of the sample reported in paper II are in good agreement with the primitive cubic lattice of the D-type. The two other samples, however, fit well into the cubic G-lattice. If the protein and the water of hydration of the protein (approximately 0.3 g/g) are subtracted, the lipid-to-water ratio will be close to 1:1 in the case of samples 2 and 3, which far exceeds the cubic gyroid phase boundary in the binary system (Fig. 2 in I), and also exceeds the lipid-to-water ratio (1:0.76) of sample 1 in Table 5. Thus, not only the amount of water, but also the amount of protein determines the $C_G \rightarrow C_D$ transition. At high protein concentrations, the preferred cubic structure seems to be the D-type, while at lower protein concentrations the cubic phase formed is of the G-type. It is seen from the phase diagram in paper II and from Table 5 that the cubic phases can swell to a higher water content when protein is present. This is also reflected by the considerable increase of the unit cell dimension. The flexibility of the cubic lattice is further stressed by the formation of cubic phases with proteins of different molecular dimensions. Table 5 includes the X-ray spacings of a cubic monoolein-BSA sample. BSA, having a considerably larger hydrodynamic dimension than lysozyme (see Table 1) also gives a larger unit cell dimension of the cubic phase. Glucose oxidase, the largest protein studied (MW 150 000) (II) has, however, a limited potential to co-operate in the formation of liquid crystalline phases. A maximum of approximately 10% (w/w) glucose oxidase can, in native form, be solubilized by the cubic phase.

TABLE 5

X-ray diffraction data of cubic 1-monoolein-protein-water samples.

Sample	d(Å)	$(h^2+k^2+l^2)$	a_0 (Å)	Cubic lattice
1:				
1-monoolein:lysozyme:				
water	96.6	3	167.3	
31.6:34.2:34.2 (w/w)	82.97	4	165.9	
	68.49	6	167.8	
	54.62	9	163.9	
	41.09	16	164.4	
	36.26	21	166.2	
	Mean value		166.1	D
2:				
1-monoolein:lysozyme:				
water	92.6	2	130.9	
42.6:11.5:45.9	65.5	4	131.0	
	53.5	6	131.0	
	41.4	10	130.9	
	37.8	12	130.9	
	35.0	14	131.0	
	32.7	16	130.8	
	Mean value		130.9	G
3:				
1-monoolein:bovine serum				
albumin:water	58.8	6	144.0	
38.5:18.4:43.1 (w/w)	50.9	8	144.0	
	37.6	14	140.7	
	32.3	20	144.4	
	30.9	22	144.9	
	Mean value		143.6	G

The cubic phase in the sunflower oil monoglyceride (Dimodan LS)-water system shown in Figure 4 does not exhibit the $C_G \rightarrow C_D$ transition at increased water content. Neither is it possible to encapsulate globular proteins into this cubic phase. Gulik-Krzywicki et al. (1984) have, however, reported the electron microscopy appearance and X-ray diffraction of a cubic sunflower oil monoglyceride (Dimodan LS)-cytochrome c-water sample. The variations in water-swelling properties between different monoglyceride preparations pointed out in 2.2.3 might explain the difference in formation of cubic protein-monoglyceride phases seen.

If the C_D -phase is formed at the interface zone between the excess water and liquid lipid phase, penetration of macromolecules into the aqueous networks would be facilitated by the wider water channels of this cubic phase. As pointed out above, incorporation of proteins requires a high flexibility of the lipid lattice with regard to lipid concentration. This demand might be better met by a lipid matrix that is able to transform into another cubic lattice almost without energy exchange.

3.1.2 Other Monoglyceride-Water Phases

In the water concentration region 5-20% (w/w) of the 1-monoolein-water system, the lamellar phase is formed at 20°C (Fig. 2 in I). This region of the phase diagram has been omitted in II due to very long time for reaching equilibrium when protein is present. This might be due to the lamellar lattice dimensions in the binary aqueous systems, with water layers of 2-6 Å (Fontell, K., personal communication). Interaction between globular proteins and the lamellar phase of phospholipid systems have been reported to depend on electrostatic interaction between protein and oppositely charged polar lipid headgroups (cf. Rand, 1975). Additionally, charged phospholipid bilayers swell to very wide water layers, due to electrostatic repulsion.

The reversed hexagonal phase is formed from the cubic phase in the 1-monoolein system at increased temperature (above approximately 90°C) when the water content exceeds 18% (w/w). This phase transition is also seen in the presence of globular proteins (II). From the DSC-results, it has been concluded that the water cylinders in the hexagonal lattice also are able to accommodate proteins.

The isotropic fluid i.e. the L2 phase, described in section 2.2.1, is formed in the monoolein system at low water content and, over the entire composition range, at increased temperature. This transition also occurs when globular proteins are present (II). On the basis of reversibility studies it was concluded that the protein molecules are distributed in the water compartments of the L2 phase.

In the ternary system monoglycerides-triglycerides-water, the L2 phase formed at 30°C can accommodate as much as 25% water (w/w) (Pilman et al., 1982). This system was used to test if a water soluble protein could be incorporated into the water compartments of the L2 phase. A water solution of cytochrome c was added to a mixture of monoglycerides-triglycerides (weight ratio 7:3). The fluid isotropic solution formed was coloured by the protein but the phase equilibria seem to be changed when the protein is present. With increasing protein concentration, decreased maximum water swelling was observed, i.e. a behaviour contrary to the cubic monoglyceride phases. With a 10% (w/w) protein solution, the maximum amount of water solubilized was 15%. It was also noticed that during equilibration of the samples, a protein stabilized interface was formed between the oil and water phases, which delayed time for equilibrium and also precipitated a part of the protein.

3.2 Biological Relevance of Protein-Monoglyceride-Water Phases

During intestinal lipolysis of dietary triglycerides, a complex mixture of free fatty acids, mono-, di- and triglycerides and bile acids occur (cf. Hofmann and Borgström, 1964). In an in vitro study of the lipolysis, Patton and Carey (1979) observed formation of different product phases during the stepwise hydrolysis of triglycerides in an intestinal-like environment. The first product phase seen was a crystalline phase, consisting of almost pure fatty acid calcium soaps, occurring on the surface of the oil droplets. As a second product phase, a viscous isotropic phase emerged, consisting of monoglycerides and free fatty acids. This phase is identical to the cubic phases in the monoglyceride systems discussed above. It has further been shown (Lindström et al., 1981) that beside cubic phases also reversed hexagonal and L2-phases are formed in lipid systems related to fat digestion.

In excess of bile salts, the lipolysis products are rapidly solubilized in mixed micells (Svärd, 1985). However, the bile acid amount in vivo is not sufficient to solubilize all lipids after a meal rich in fats (Borgström,

1985), which implies that the liquid crystalline phases exist in vivo. A physiological role of the cubic phases might be related to the bicontinuous three-dimensional structure, facilitating transport and fusion. Since lipase and water should be free to diffuse through this medium, the bicontinuity and the incorporation properties of the cubic phase discussed above are probably an important feature for the lipolysis process (Fig. 5).

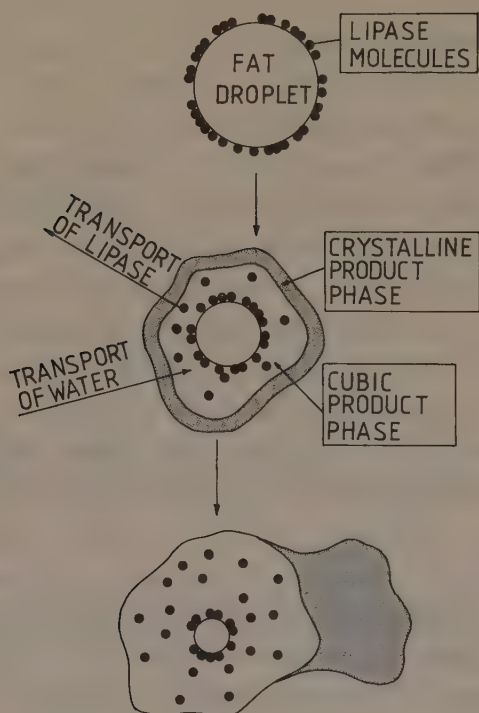


Figure 5. A simplified illustration of the events taking place during the lipolysis of a triglyceride droplet. The first product phase, a crystalline crust, which initially forms a "skin" around the oil droplet, bursts and the cubic phase emerges. The product phases must allow diffusion of lipase molecules as well as of co-substrate and products (interpretation of data from Patton et al., 1985).

The possibility of absorption of native insulin in the form of an insulin-monoolein-water cubic phase through the intestinal wall, has been studied in collaboration with professor John Wahren at the Department of Clinical Physiology at Huddinge Hospital, Stockholm, Sweden (Wahren, J. and Ericsson, B., to be published). Healthy fasting persons were given a bile salt dispersion of the cubic monoolein-insulin phase intraduodenally, and the glucose content in blood was followed. No decrease in glucose concen-

tration could be seen. The most plausible explanation for the observed behaviour is that the cubic phase does not protect the protein against proteolytic cleavage. The result indicates that the cubic phase cannot be used for absorption of native insulin through the intestinal wall. It also indicates that probably no water-soluble native proteins can be absorbed with the aid of the cubic phase.

The cubic monoolein phase has also been found to induce membrane fusion (Tilcock and Fisher, 1982), which means that the cubic phase, when containing proteins, might act as a vehicle for proteins through cell membranes.

3.3 Technological Relevance of Protein-Monoglyceride-Water Phases

The possibility to immobilize native proteins at high concentration in a well-defined naturally occurring lipid matrix might be utilized in technological applications. Since it has been shown (II) that lysozyme can diffuse from the cubic phase to an aqueous environment, the monoolein-lysozyme phase might be used as an antimicrobial system.

Several applications of monoglyceride mesophases should be possible within the food field. Since both hydrophilic and lipophilic substances are soluble in the mesophases, they may act as carriers for different types of active agents. Both the L2-phase and the cubic phases might be used as vehicles for protein antioxidants, e.g. superoxide dismutases, in fat based products. The rheological properties of the cubic phase allow easy formation of a thin film on surfaces in for example a bioreactor or on a separation membrane, making possible encapsulation of enzymes as one alternative to conventional immobilization in continuous biotechnological processes.

4 INTERACTIONS BETWEEN GLOBULAR PROTEINS AND WATER SOLUBLE POLAR LIPIDS

Most of the reports found in literature concerning protein-polar lipid interactions in dilute systems deal with the interaction between SDS and globular proteins at low and intermediate temperature (cf. reviews by Steinhardt and Reynolds, 1969; Steinhardt, 1975; Lapanje, 1978; Makino, 1979). SDS is known to bind to most proteins in a non-specific cooperative manner, thereby destabilizing the native conformation (cf. 2.1.4). Other long-chain alkyl sulphates as well as alkyl sulphonates, benzene sulphonates, phosphates and soaps may behave in a similar way. The extent of unfolding depends both on chain length and on the nature of the ionic group. The interaction is reported to be restricted to the monomeric form of the

amphiphile. Two proteins, serum albumin and β -lactoglobulin, are known to have high-affinity binding sites for low molecular weight amphiphiles (cf. Table 1). Highest affinity is reported for the anionic ones.

Nonionic amphiphiles are frequently used for membrane solubilization (cf. Helenius and Simons, 1975). No interaction with native water-soluble proteins, with the exception of serum albumin and β -lactoglobulin, have been reported (Clarke, 1975).

The cationic amphiphiles seem to exhibit an intermediate action on water-soluble proteins. Reports in the literature indicate a co-operative interaction with proteins, but with less affinity and thus with less perturbation of the folded state, compared to the effect of anionic ones (Jones et al., 1973; Nozaki et al., 1974; Jones et al., 1975; Tanford, 1980). Results indicating differences in complexes formed between proteins and cationic compared to anionic amphiphiles regarding the solubilization of hydrophobic probes have also been reported (Birdi and Steinhardt, 1978).

Proteins are important for the quality characteristics of several major foods. The "functionality" of proteins in food systems (cf. Kinsella, 1982) derives from the physico-chemical properties of the protein, which are influenced by interaction with other food components during processing and storage.

Solubility is an important property of food proteins per se, but also many other technological properties, such as foaming and emulsification, are related to the initial solubility of the protein. Gelation is fundamental for the texture of many food products (e.g. sausages, bread). Viscosity is relevant in food products where thickening is crucial, but is also of practical significance in process design (e.g. pumping). Emulsification and foaming properties are directly related to the surface tension of present proteins and lipids. Enzymatic activity may be classified as a technological property in the sense that it can be used to alter physico-chemical properties of food components and thereby affect quality and processing characteristics (Feeney and Whitaker, 1977).

During processing of food products, its components are sustained to substantial external stress, which influence structure and properties of the components. Heating and shearing are common treatments of food systems.

This part of the thesis will discuss physico-chemical changes of various protein-amphiphile complexes in relation to different external stress.

4.1 Thermal Treatment

Thermal treatment is the most common technical unit operation in food production. The aims of increased temperature are manifold. Primarily, a change in texture of the product is desired, thereby affecting nutrition and organoleptic qualities (e.g. increased water holding capacity). A reorganization of protein structure is, in this case, inevitable. In other cases the aim is merely to increase the shelf-life of the products by blanching, pasteurization or sterilization. In biotechnological processes based on enzymatic production, an increased operational temperature is often desired in order to optimize the yield and to prevent bacterial growth. In these latter examples, protein unfolding or aggregation may be undesired.

4.1.1 Denaturation

In principle, a denaturated protein can be refolded to the native state on removal of the denaturant. In all practical cases, however, the reversibility is limited due to different inter- and intrachain interactions (cf. Pain, 1983). Paper III describes the heat denaturation process of ovalbumin, which has a reversibility close to zero %. Paper IV deals with the heat denaturation of lysozyme, which is reversible to at least 80% at the experimental conditions used. Due to the limitation in reversibility, the thermal history of protein-based products will influence the technological properties in subsequent processes.

The strong denaturing effect of anionic surfactants on proteins at room temperature earlier reported was also observed on the model proteins used here. 40 mM SDS changes the protein conformation already at low temperature (cf. 2.1.4). The final reorganized structure is unsusceptible to heat. An exception was, however, found for ovalbumin at acidic pH-values (III).

The cationic amphiphiles cause minor or no refolding of the model protein structures at low temperature, but alter the thermal denaturation process. In paper III the effect of HPC on the thermal denaturation of ovalbumin is described. At increased temperature HPC has a destabilizing effect on ovalbumin. The degree of unfolding at 95°C, however, appears to be lowered compared to pure ovalbumin. The thermal denaturation of the ovalbumin-HPC

complex is, contrary to pure ovalbumin, highly reversible on cooling. This might be explained by decreased inter- and intrachain interactions at high temperature, facilitating re-formation on cooling.

A more thorough investigation on the effect of cationic low-molecular weight amphiphiles on a globular enzyme (lysozyme) at different temperatures confirms that cationic amphiphiles are able to suppress thermal unfolding of proteins (IV). Catalytic activity of lysozyme was, however, not depressed by the cationic amphiphiles at predenaturation temperatures - even an increased activity was registered at the temperature optimum (Fig. 5, IV). The thermal denaturation of lysozyme is dependent on the association state of the amphiphile. The micelle forming surfactants HPC and DAC (cf. Table 2) suppress thermal unfolding at concentrations near and above their cmc. As was noticed for ovalbumin, the post-denaturation state of lysozyme seems to exhibit a higher degree of order in the presence of cationic surfactants.

Increased salt concentration increases the effect of amphiphiles on lysozyme. Thermal denaturation of lysozyme in HPC at concentrations above cmc in saline of different concentrations is seen in Figure 6. At these relatively low electrolyte concentrations, the most significant effect of salt arises from the Debye-Hückel shielding. As the ionic strength is increased, the stability of lysozyme against thermal stress decreases, and the denaturation pattern becomes similar to the one seen on ovalbumin with HPC. Hydrophobic association is promoted with decreased electrostatic repulsion, and a stronger interaction between the protein and the amphiphile can occur. In a recent paper, Jones et al. (1984) have shown that hydrophobic binding of SDS to globular proteins increases with increased ionic strength. Generally it is assumed that since increased ionic strength decreases the cmc, the interaction between micelle forming surfactants and a protein also should decrease (Reynolds and Tanford, 1970).

A liposome forming (class 2) surfactant, HLDC, has also been investigated. This compound has a somewhat different influence on protein conformation and unfolding, compared to the micelle forming surfactants. HLDC does not induce any effect on lysozyme conformation at predenaturation temperature. The thermal unfolding, however, is suppressed at sufficiently high concentrations - an effect which is proportional to the HLDC concentration (Fig. 4 in IV).

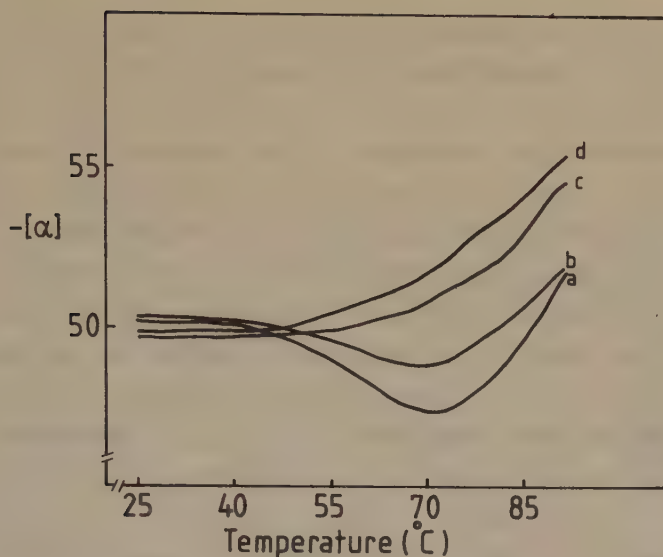


Figure 6. Specific optical rotation of lysozyme (10 g/l) in HPC (3 g/l) at pH 5. Ionic strength was varied by adding NaCl. Curve a: no addition; curve b: 0.01 M NaCl; curve c: 0.03 M NaCl; curve d: 0.05 M NaCl. Specific optical rotation of pure lysozyme at pre-denaturation temperatures is not influenced by added NaCl at these concentrations. The aggregation temperature is, however, decreased by 5°C.

The specific optical rotation (OR) curves versus temperature shown in paper IV (Fig. 2) show a decrease in levorotation at intermediate temperature, which might indicate an increased protein-amphiphile interaction. The effect is reversed on cooling. The hydrophobic interaction is known to become stronger with increasing temperature in the temperature interval discussed here, although its rate of increase decreases with temperature (cf. Ben Naim, 1980). Thus, it might be assumed that hydrophobic interaction between the protein and the hydrocarbon moiety of the surfactants increases as temperature increases, and that the hydrophobic parts of the protein thereby will be sheltered from the aqueous medium. Due to the temperature dependence of the hydrophobic interaction it should contribute to the thermal stability of proteins. An increased hydrophobic interaction, which not causes refolding of the protein structure, might be assumed to increase the thermal stability of a protein.

The sensitivity of the lysozyme-cationic amphiphile complexes to different experimental conditions demonstrates the importance of a balancing of attractive and repulsive intermolecular forces for the complex formation. At conditions favouring the hydrophobic force (amphiphile concentration near cmc, intermediate temperature; increased ionic strength) the repulsive force, acting between charges of equal sign, will be overcome. A cooperative association, analogous to micelle formation, of the amphiphile to the solvent accessible hydrophobic surfaces of lysozyme will be facilitated. This process might be visualized as an "emulsification" of the protein by the surfactants. This interaction does not cause refolding of the secondary structure of lysozyme at room temperature (IV, Fig. 1). Interaction at low surfactant concentration (below cmc) will be discussed in the next section.

An attempt was made to correlate native structural characteristics of proteins to the structural response against the investigated cationic amphiphiles. The influence of HPC and DAC on seven proteins with quite different secondary unit composition in native state (Table 6) were examined by circular dichroism and the results are summarized in Table 7. The effect on trypsin conformation is shown in paper VI. DAC, at concentrations below the cmc, exhibits weak influence on the conformation of all the proteins in Table 7, while the structure of trypsin is altered by cationic amphiphiles already at concentrations well below their cmc. This alteration, however, results in both increased stability and increased activity of trypsin (VI). This effect will be further discussed below. At DAC and HPC concentrations above cmc, different patterns are seen. Myoglobin, β -lactoglobulin, conalbumin and concanavalin A undergo a refolding in the presence of the amphiphiles, while the conformation of superoxide dismutase, cytochrome c and papain are not influenced. The response to heat of the refolded structure of conalbumin is shown in Figure 7. The formed structure is unsusceptible to heat, as is the case with proteins refolded by SDS.

Both an all- α protein (myoglobin) and an all- β protein (concanavalin A) are thus unfolded by the cationic amphiphiles, while another β -rich protein (superoxide dismutase) is not affected. No correlation between the secondary structure and the effect of the cationic surfactants can thus be found for these proteins. The same applies for proteins which contain both α - and β -structure. The results point to the diversity in the effects of ionic

TABLE 6

Selected molecular properties of the proteins used in the circular dichroism measurements.

Protein	pI	MW	α -helix (%)	β -sheet (%)	β -turn (%)
Conalbumin ¹ (egg white)	6.8 ⁵	77000 ¹⁰	28	32	-
Concanavalin A ² (Jack beans)	7.0-7.2 ⁶	55000 ¹¹	3	64	22
Cytochrome c ² (horseheart)	10.0 ⁵	13000 ⁵	43-48	9	23
β -Lactoglobulin ³ (milk)	5.1 ⁷	18300 ⁷	16	18	-
Myoglobin ⁴ (sperm whale)	7.0 ⁵	17000 ⁵	79	0	12
Papain ⁴ (papaya latex)	8.7 ⁸	23000 ⁸	28	9	14
Superoxide dismutase ² (bovine erythrocytes)	5.0 ⁹	33000 ⁹	0	52	26-35

¹Tan (1971) (secondary structure)⁷Eigel et al. (1984)²Brahms and Brahms (1980) (secondary structure)⁸Liener (1974)³Hillqvist Damon and Kresheck (1982) (secondary structure)⁹Bannister et al. (1971)⁴Hennessey and Johnson (1981) (secondary structure)¹⁰Warner and Weber (1953)⁵Mahler (1969)¹¹Kalb and Lustig (1968)⁶Agrawal and Goldstein (1967)

TABLE 7

The effect of DAC and HPC on the conformation of the proteins in Table 6 expressed as the mean residue ellipticities (θ) at 222, 214 and 208 nm. The last column gives an estimation of the effect (0: no effect; + increased ordered structure; - decreased ordered structure). CD-spectra were recorded in the wave-length region 280-195 nm (as described in paper IV). Circular dichroism in the far UV-region yields information about the back-bone conformation of proteins. The minima at 208-210 nm and at ~ 222 nm originate from α -helical structure, while pure β -structure gives a minimum at 214-216 nm. A polypeptide chain in aperiodic form exhibits a minimum in the wave-length region 193-200 nm (cf. Brahms and Brahms 1980).

Protein (0.2%)	Additive	θ_{222}	$\theta_{214-216}$	$\theta_{208-210}$	$\lambda_{\min}$	$\theta_{\min}$	Effect
Conalbumin pH = 5.0		-7100	-8600	-8300	209-210	-9200	
	DAC (4.2 mM)	-7400	-9200	-9000	209-210	-9700	0
	DAC (16 mM)	-8000	-9200	-11700	208	-11700	+
	HPC (2,7 mM)	-7400	-8900	-10700	208	-10700	+
Concanavalin A pH = 5.0		-6600	-3300	+1100	225	-6900	
	DAC (4.2 mM)	-6500	-3600	+250	223	-6700	0
	DAC (16 mM)	-8500	-7600	-7400	218-222	-8500	+
	HPC (2.7 mM)	-8600	-7500	-6600	218-222	-8600	+
Cytochrome c pH = 5.0		-10300	-8500	-7600	222	-10300	
	DAC (4.2 mM)	-10200	-8300	-7500	222	-10200	0
	DAC (16 mM)	-9900	-8700	-9000	222	-9900	(-)
	HPC (2.7 mM)	-10400	-8500	-7600	222	-10400	0

TABLE 7 cont.

Protein (0.2%)	Additive	Θ_{222}	$\Theta_{214-216}$	$\Theta_{208-210}$	λ_{min}	Θ_{min}	Effect
β -Lactoglobulin pH = 4.0		-5300	-6100	-4300	216	-6100	
	DAC (4.2 mM)	-5700	-6500	-4600	216	-6500	0
	DAC (16 mM)	-11500	-12100	-15500	208	-15500	+
	HPC (2.7 mM)	-10300	-11000	-13800	208	-13800	+
Myoglobin pH = 5.0		-21300	-19100	-19100	222	-21300	
	DAC (4.2 mM)	-21100	-18900	-19300	222	-21100	0
	DAC (16 mM)	-13700	-14000	-17200	208	-17200	-
	HPC (2.7 mM)	-15300	-15300	-18100	208	-18100	-
Papain pH = 5.0		-7800	-7900	-9200	208	-9200	
	DAC (4.2 mM)	-7800	-7900	-9200	208	-9200	0
	DAC (16 mM)	-8100	-8300	-9500	209	-9800	0
	HPC (2.7 mM)	-7900	-8200	-9400	209	-9500	0
Superoxide dismutase pH = 4.0		-4400	-8000	-8800	210	-9000	
	DAC (4.2 mM)	-4500	-8200	-9100	210	-9200	0
	DAC (16 mM)	-4300	-8000	-8700	210	-9100	0
	HPC (2.7 mM)	-4500	-7900	-8600	210	-8900	0

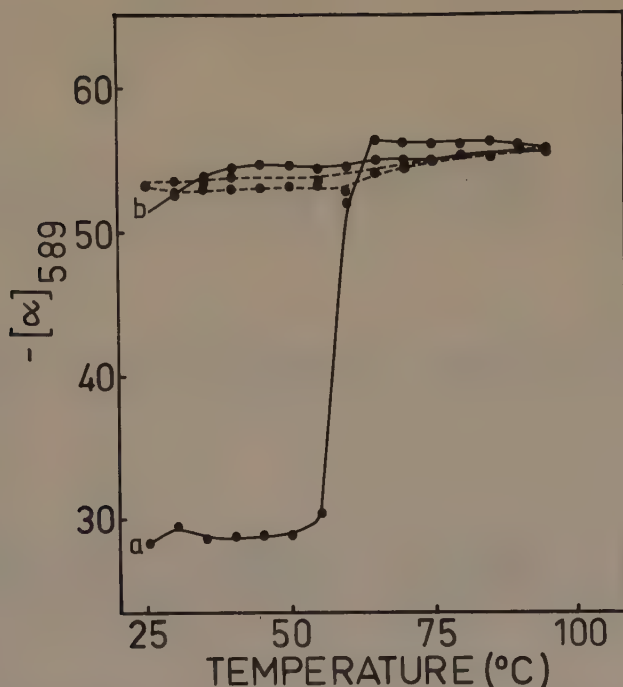


Figure 7. Specific optical rotation of a) conalbumin and b) conalbumin in HPC (weight ratio 0.40 g/g) at pH 5.

— : heating 1°C/min
 ---- : cooling 1°C/min

amphiphiles on proteins. However, the proteins with high isoelectric point are more resistant to refolding by the cationic surfactants. It can thus be assumed that the balancing of the attractive hydrophobic force by electrostatic repulsion is one important factor which determines the state of the complex formed.

4.1.2 Thermal Aggregation

Thermal treatment of proteins results in an increased tendency for protein-protein interactions (i.e. aggregation), which influence several major technological properties, such as gel formation and solubility. Aggregation phenomena are highly dependent on conditions effecting protein-solvent interactions (pH, ionic strength, type of counterion, co-solutes), as well as on kinetic parameters (Hegg, 1978).

The solubility of proteins is affected by cationic amphiphiles already at concentrations well below cmc, while generally no effect on protein conformation was found at these concentrations (IV; Table 7). Cationic amphi-

philes increase the solubility of proteins at the acidic side of pI (III, IV). The interaction between the protein and the cationic amphiphiles in this concentration range thus seems to be of electrostatic origin, i.e. the polar group of the amphiphile interacts with negatively charged groups on the protein, thereby increasing the positive net charge of the protein. On the alkaline side of pI this will lead to precipitation. Redissolution of the precipitate will start as the ratio of amphiphile-to-protein is increased above the amount of opposite charges on the protein. Anionic amphiphiles will affect solubility in the opposite direction (cf. Birdi, 1973).

At increased temperatures, additional forces have to be accounted for in order to elucidate the aggregation behaviour. Of major concern are various kinds of attractive forces developing during the denaturation transition. Generally speaking, if the net attractive forces set free on denaturation are larger than the electrostatic repulsive forces, this will result in protein aggregation. Consequently, increasing the electrostatic repulsion by bound cationic amphiphiles at the acidic side of pI, can result in a situation where protein-protein interactions are prevented even on heat denaturation (III, IV).

Gelation is a special case of aggregation, where a three-dimensional network with high water retention is achieved. The pre-requisite for thermal gel formation of separated proteins has been described by Hegg (1982). Gels are formed preferentially at conditions close to solubility, i.e. at conditions where electrostatic repulsion is strengthened. Among the model proteins used in this thesis, ovalbumin gels at low concentration, while trypsin and lysozyme only gel at high concentrations. Globular proteins usually gel only under thermal stress. An exception from this principle was found for trypsin. A gel was formed at room temperature on incubation for 12 hours of a 5% (w/w) trypsin solution at pH 3.0. Furthermore, the gel was tixotropic (unpublished results).

As thermal gel formation requires a careful balance between attractive and repulsive forces, it can be influenced by ionic surfactants. A positive effect by SDS at increased ionic strength, on the thermal gelling behaviour of ovalbumin at the alkaline side of pI has been described (Hegg et al., 1979). A similar effect might thus be expected by adding a cationic amphiphile to a protein at the acidic side of pI. This has been verified by experiments on HPC and ovalbumin (unpublished results). The possibility of regulating thermal gel formation of ovalbumin is smaller on the acidic side of pI, since titration of the carboxylate groups rapidly changes the net charge of the protein, making the pH-interval for gelation extremely narrow.

Aggregation must also be considered when preserved biological activity of a protein is required during or after thermal treatment, since aggregation can markedly decrease the activity. In paper IV increased enzymatic activity at elevated temperatures is observed for lysozyme in the presence of cationic amphiphiles. In the pH interval 5-9, lysozyme is known to dimerize (Sophianopoulos and van Holde, 1964), a process which should be facilitated by heating. Norton and Allerhand (1977) have shown that the active site of lysozyme is involved in this association process. Increased electrostatic repulsion attained by the cationic amphiphile should diminish the dimerization process and thus increase lysozyme activity.

4.2 Interfacial Films

The stabilizing of interfacial films in colloidal food systems, such as emulsions and foams, are provided by present proteins and/or polar lipids. Film formation and film properties of these components differ though. An important difference is the ability of polar lipids to reduce surface tension to a lower value than most proteins do. Furthermore, the adsorption-desorption kinetics for the two specimens differ. Soluble low-molecular-weight amphiphiles show a high rate of exchange at the interface, while proteins have a more complex behaviour. More multiple anchoring at the surface for the protein results in a lower rate of desorption (cf. MacRitchie, 1978). According to MacRitchie (1985), the rate of desorption of proteins is dependent on an interplay between molecular weight, interfacial pressure and adsorption kinetics.

The adsorption of macromolecular species at interfaces is time-dependent, which has been assigned to different rate determining steps (see e.g.

MacRitchie, 1978; Tornberg, 1978; Graham and Phillips, 1979). Three stages can be identified:

1. At low surface concentration the adsorption rate is determined by the diffusion of the macromolecules to the interface. The surface tension decrease ($\Delta\gamma$) is related to the diffusion coefficient (D) by the equation

$$\Delta\gamma = 2C_p kT \left(\frac{Dt}{\pi}\right)^{\frac{1}{2}}$$

where C_p is the bulk protein concentration.

2. At increased surface concentration the already adsorbed molecules give rise to a positive energy barrier to adsorption. The adsorption rate will no longer be diffusion controlled, but will be determined by the rate of penetration of molecules or segments into the surface layer.
3. Rearrangement of the conformation of the adsorbed molecules takes place, lowering the surface tension over a prolonged period of time. Graham and Phillips (1979) have reported relaxation times of several hours.

In the report by Graham and Phillips cited above, the rate of the penetration and rearrangement processes during protein adsorption at the air-water interface were analyzed according to the first-order equation

$$\ln \left(\frac{\gamma_t - \gamma_{eq}}{\gamma_0 - \gamma_{eq}} \right) = \frac{t}{\tau}$$

where γ_{eq} , γ_0 and γ_t are the surface tension values at equilibrium, at zero time and at time t , respectively. τ is the relaxation-time and equals the reciprocal of the rate constant. From experimental data on surface tension and surface concentration (Γ), two different relaxation times were identified: τ_1 related to the penetration process, i.e. when both γ and Γ are changing, and τ_2 related to the rearrangement process, i.e. γ is decreasing but Γ is constant.

In this work, surface tension has been measured by the drop-volume technique according to Tornberg (1977, 1978), with the automatization procedure by Arnebrant and Nylander (1985) employed. This method allows surface tension to be measured also at short times, since the formation of a fresh

surface is accomplished within a few seconds. During the surface tension decay (from formation until detachment of the drop) the surface of the drop expands. According to Tornberg and Lundh (1981) this does not introduce any error in measured time-dependence of the surface tension decay. Adsorption as measured by this technique can be considered analogous to adsorption at an interface created without further disruption in a foam or an emulsion.

4.2.1 Surface Tension of Ovalbumin

Figure 8 shows the surface tension decay with time of ovalbumin (pH = 4.0) at different concentrations at the air-water interface. The different rate determining steps, the diffusion controlled (1), and the penetration (2) and rearrangement (3) processes, evaluated according to Graham and Phillips (1979), is indicated by arrows. The overall adsorption rate is dependent on the subphase protein concentration. For the highest protein concentration, the diffusion step is too fast to be determined accurately, and only the penetration and rearrangement processes are indicated in Figure 8. It has been shown that the adsorption rate also is dependent on the flexibility of the protein as well as on pH and ionic strength, and Blank et al. (1975) have shown an inverse relation between adsorption rate and apparent net charge of the adsorbed molecules (cf. curves 1 in Fig. 12, A and B).

The equilibrium surface tension reduction at the air-water interface as a function of subphase protein concentration at pH = 4.0 is shown in Figure 9. Surface tension reduction is expressed by

$$\Delta\gamma_{eq} = \gamma_0 - \gamma_{2000}$$

where γ_0 is the surface tension at zero time, taken as the tension of pure water against air (72.4 mN m^{-1} at 23°C). γ_{2000} is the surface tension for the protein solution after 2000 seconds. After this period of time the decline in surface tension is small and a "near equilibrium" surface tension is reached. At extended time, surface ageing might induce an error in the measured value. The maximum $\Delta\gamma$ of ovalbumin (23 mN m^{-1}) is reached at a bulk concentration of about 0.6 g/l . No plateau region in the lower concentration range, as reported by Bull (1972), has been found in the present investigation. A two-step surface pressure isotherm has been attributed to a transition of a surface denaturated protein film to a film containing native molecules (Bull, 1972; Graham and Phillips, 1979). This transition is supposed to occur at constant surface pressure. The $\Delta\gamma$ isotherm in

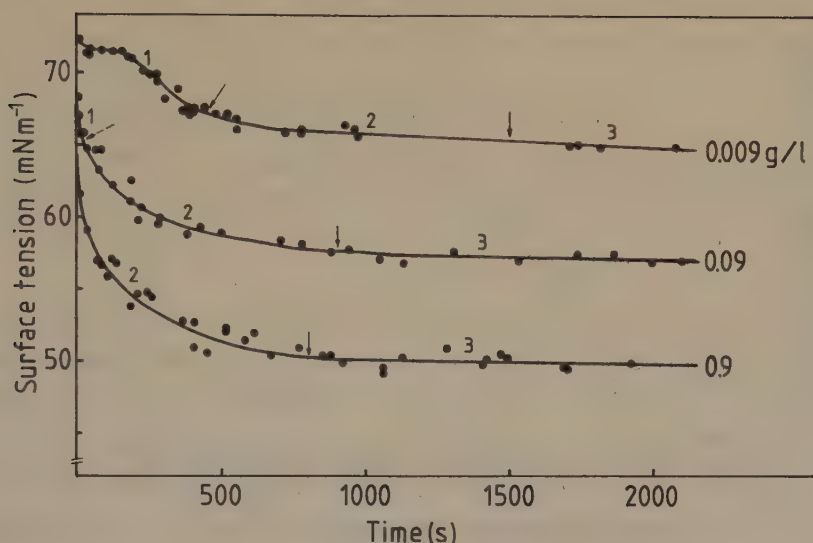


Figure 8. Time-dependence in surface tension reduction of ovalbumin at different concentrations. The diffusion (1), penetration (2) and rearrangement (3) determined steps are indicated by arrows. The diffusion controlled adsorption from the 0.9 g/l ovalbumin solution is too fast to be determined accurately and is omitted. The uncertainty in separating the diffusion step from the penetration step for adsorption from 0.09 g ovalbumin/l is indicated by a broken line arrow.

Figure 9 is more consistent with the view that no native molecules, in mixture with surface denaturated ones, are retained at the surface. In accordance with Gibbs's equation of adsorption, surface tension decreases with increasing concentration until its optimum value has been reached. With this type of surface tension isotherm, the compactness of the proteins in the surface film is supposed to be a function of surface pressure (MacRitchie, 1978).

The inconsistency between the two surface tension isotherms might be found in the different methods employed. In the work of Bull (1972), surface adsorption was measured by the Wilhelmy plate technique. To create a clean interface ($\Pi = 0$) the pouring technique was used. By initially creating a larger surface than during the actual measurements, surface adsorption and surface denaturation might be facilitated, which will give a too high value in surface pressure at low bulk concentrations. In the drop-volume technique used in the present work, the cleaning of the interface is carried out

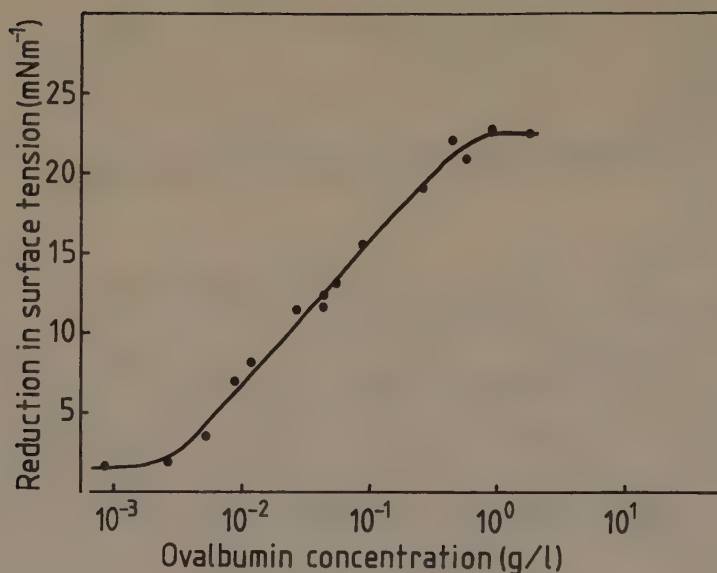


Figure 9. Equilibrium surface tension reduction isotherm of ovalbumin, pH 4.0 at 23°C. The surface tension value reached after 2000 seconds is taken as the equilibrium value.

by rapidly forming droplets, with the detached volume corresponding to γ_0 , in between those to be measured. As pointed out by Tornberg (1978) this procedure will create an initially clean surface, as long as the rate of the surface adsorption process is negligible compared to the time of formation of both the cleaning and the measuring drops (~ 20 s). This holds true in the case of protein adsorption at low subphase protein concentrations (cf. Figure 8). Differences in the "clean" surface at zero time thus can explain the different forms of the two isotherms of ovalbumin reported.

4.2.2 Surface Tension of Globular Protein-Polar Lipid Mixtures

The surface adsorption of proteins in mixture with low molecular weight surfactants gives rise to different types of surface tension isotherms. Interfacial adsorption will be competitive and determined mainly by adsorption kinetics and the surface tension depression effect of each component. Also mutual interaction in the bulk solution and in the adsorbed layer is of importance for interfacial film formation. Garcia Dominguez et al. (1981) have shown that the surface tension reduction of lysozyme and insulin at pH 3.5 decreased when an anionic surfactant (SDS) was added. This

was explained by precipitation of the protein in the bulk solution (cf. 4.1.2). Buckingham et al. (1978) found a strong synergistic lowering of the surface tension of a mixed solution of poly-L-lysine and SDS at concentrations below precipitation, micellization or complex formation in the bulk solution. Similar behaviour was observed in mixtures of low-molecular weight amphiphiles of opposite charges (Lucassen-Reynders, et al., 1981). This effect was assigned to the formation of electroneutral complexes in the interface film.

The surface tension reduction at the air-water interface at adsorption from a water-phase containing a mixture of negatively net-charged proteins and nonionic or anionic amphiphiles is reported in paper V. Depending on the ratio of low molecular weight amphiphile to protein, and on the interaction between the two components, different patterns are seen. In the binary system monocaproin and ovalbumin, the resulting equilibrium surface tension is determined by the concentration ratio of the components. No synergistic effect was observed. The behaviour thus follows the Gibbs's equation of adsorption, with the component giving the lowest surface tension displacing the other at the surface. This indicates that no molecular interaction takes place either in the bulk solution or in the adsorbed film.

Ionic amphiphiles influence the interfacial behaviour of mixed protein-polar lipid systems in a more complex way than the nonionic amphiphile does (V). The specific high affinity binding (cf. Table 1) of SDS to serum albumin (Fig. 1, V) does not influence the surface tension reduction of this protein, although it is known that the specific binding of anionic amphiphiles increases the stability of serum albumin against denaturation (cf. Gumpen et al., 1979; Peters Jr., 1985). At SDS concentrations above those required to saturate the specific binding sites of BSA, plateau regions in the surface tension reduction isotherm are seen. Constant surface tension at increased SDS concentration has been interpreted as due to co-operative binding of the amphiphile to the protein (V), resulting in near constant monomer concentration in the bulk solution (cf. Steinhardt and Reynolds, 1969). The occurrence of two plateau regions in the BSA-SDS isotherm might reflect step-wise binding of SDS to the different domains of BSA. The plateau value in surface tension reduction extends over a broader concentration range in the ovalbumin-SDS isotherm shown in paper V (Fig. 2). Since no specific binding of SDS occurs to this protein, SDS monomer con-

centration will be higher, facilitating unspecific interaction at lower total SDS concentration.

In addition to the $\Delta\gamma_{eq}$ isotherms presented in paper V, Figure 10 shows the equilibrium surface tension reduction of 21 μM ovalbumin ($0.9 \text{ g} \cdot \text{l}^{-1}$) as a function of the concentration of the cationic amphiphile HPC (experimentals as in V). The measurements have been carried out at pH 4.0, i.e. at the acidic side of the isoelectric point of the protein, in order to avoid precipitation. $\Delta\gamma_{eq}$ of pure HPC in double distilled water is shown by the dashed line in Figure 10. Cmc of HPC is 0.9 mM, which is in the same range as SDS in paper V. The synergistic effect on surface tension noted in paper V (Fig. 2) is more pronounced in the mixture containing the cationic amphiphile. Even at the lowest HPC concentration examined, 10^{-3} mM, corresponding to about 0.05 mole HPC per mole ovalbumin, $\Delta\gamma_{eq}$ increases by 4 mN m^{-1} to 27 mN m^{-1} . Pure HPC has a negligible surface tension reduction ability at this concentration. The $\Delta\gamma_{eq}$ value of the mixture is almost constant over more than a 10-fold increase in HPC concentration. At HPC-to-ovalbumin ratios as low as 0.05 to 0.5 mole/mole it is not plausible that interaction, which can affect surface adsorption, occurs in bulk solution. The observed increase in $\Delta\gamma$ therefore probably arises from interaction in the adsorbed surface film. A pronounced time-dependence in surface tension was seen in this concentration range (Fig. 12B), indicating surface modification processes proceeding over longer time periods than in the pure protein film.

A further increase in HPC concentration leads to an increase in $\Delta\gamma_{eq}$ analogous to the increase in $\Delta\gamma_{eq}$ by pure HPC. Well below the cmc a second plateau region is reached at 31.7 mN m^{-1} , which is slightly above the surface tension plateau value of pure HPC. At HPC concentrations below cmc, an electrostatic interaction to globular proteins, only influencing intermolecular repulsion, occurs in bulk solution (cf. 4.1.2). It is, however, not possible to unambiguously assign the plateau region found in the surface tension isotherm to ovalbumin-HPC interaction in the bulk solution. The constant level of $\Delta\gamma_{eq}$ might arise either from a constant monomer concentration of HPC (i.e. binding) or from attained minimum in surface tension.

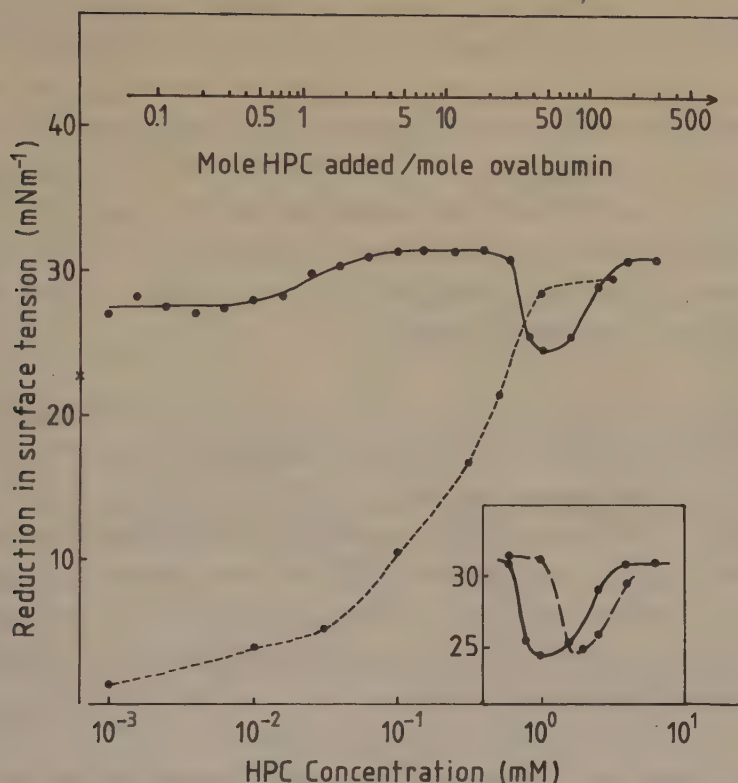


Fig. 10. Equilibrium surface tension reduction isotherms of HPC (dashed line) and of 21 μM ovalbumin in HPC at 23°C. The value for pure ovalbumin is indicated on the ordinate. The inserted shows the surface tension reduction of 21 μM and 42 μM ovalbumin in HPC of concentrations in the vicinity of cmc.

The marked minimum in $\Delta\gamma_{\text{eq}}$ seen in the HPC concentration range 0.8 to 2.5 mM probably reflects an increased HPC-ovalbumin interaction in the bulk solution, leading to formation of a highly charged hydrophilic complex. The protein will thus not contribute to the surface tension reduction of the mixture and $\Delta\gamma_{\text{eq}}$ will decrease. At increased HPC concentrations surface tension reduction is governed by HPC only. The minimum in $\Delta\gamma_{\text{eq}}$ is dependent on ovalbumin concentration. The $\Delta\gamma_{\text{eq}}$ isotherms of 21 and 42 μM ovalbumin in HPC of concentration 0.6 to 6 mM is shown in the insert in Figure 10. The minimum in $\Delta\gamma_{\text{eq}}$ is shifted 0.66 mM by the difference in protein concentration. This displacement measures the saturation binding of HPC to ovalbumin to be 30 mole HPC per mole ovalbumin.

In IV, differences in thermal unfolding of a protein in the bulk solution was found near the cmc of the amphiphile, irrespective of protein concentration (1-5%). According to the surface tension measurements, the discontinuity is found at or above the cmc. Assuming a surface protein concentration of $0.3 \mu\text{g}/\text{cm}^2$ (DeFeijter et al., 1978) less than 0.5% of the protein present in the bulk solution will be located at the interface. The discontinuity in surface tension will thus not be apparent until the complex formation is nearly complete, providing that the complex formed is the least surface active "component" present.

The pre-treatment of proteins, which will be used for interfacial film formation (e.g. as emulsifier), is of great importance. Thermal denaturation of protein with subsequent aggregation will decrease the surface activity (Barton et al., 1986) and also increase the film thickness at emulsification (Walstra, 1983). Denaturation with preserved solubility, however, will increase the surface activity (Graham and Phillips, 1976) and also give a thinner surface film. Such increased solubility can be achieved by ionic amphiphiles at low ratio.

Figure 11A and B shows the surface tension by pure monocaproin and HPC as a function of time. The main γ lowering occurs during the first seconds, although a pronounced time-dependence can be seen by HPC, especially at low bulk concentrations. Presence of impurities (homologues or hydrolysis products) might cause considerable time-dependence in surface tension reduction by surfactants, even over periods of days (Kloubek and Neumann, 1969). On the other hand, highly purified ionic surfactants also show some time-dependence at concentrations well below cmc.

The time-dependence of γ of some mixing ratios of ovalbumin-monocaproin and ovalbumin-HPC is shown in Figure 12A and B. The protein concentration is kept constant at $0.9 \text{ g} \cdot \text{l}^{-1}$. The assumption made in V that monocaproin can replace the protein at the surface film, is consistent with the kinetic curves. These change gradually from a shape typical of the pure protein to a shape typical of pure monocaproin, as monocaproin concentration increases. In the transition region surface films containing both components form. A considerable lowering of γ , due to the shorter diffusion time by monocaproin compared to the protein, is seen within the first few seconds. In addition, a time-dependent surface tension decrease, due to the protein, is observed.

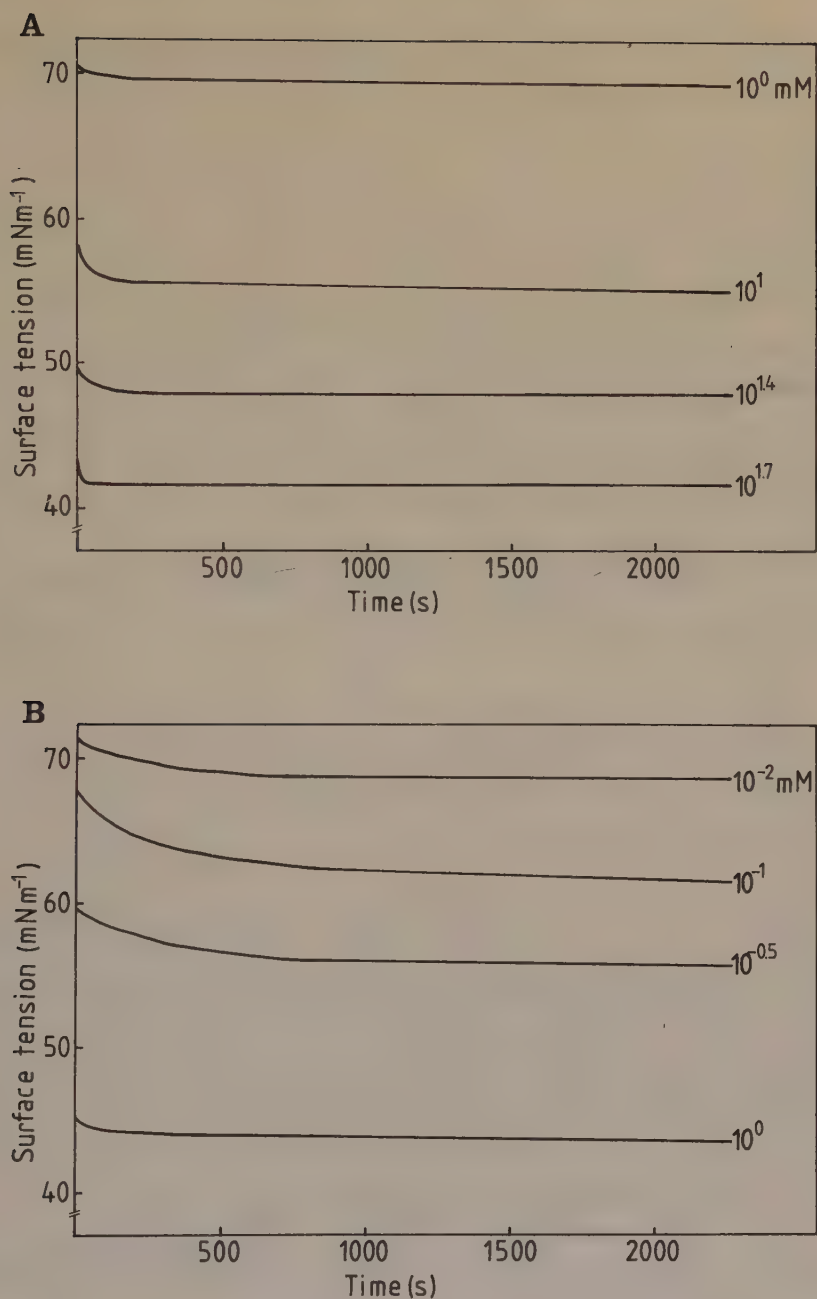


Figure 11. Time-dependence of surface tension of monocaproin (A) and HPC (B) of different concentrations. Measuring points have been omitted for clarity (measuring point scattering as in Fig. 8).

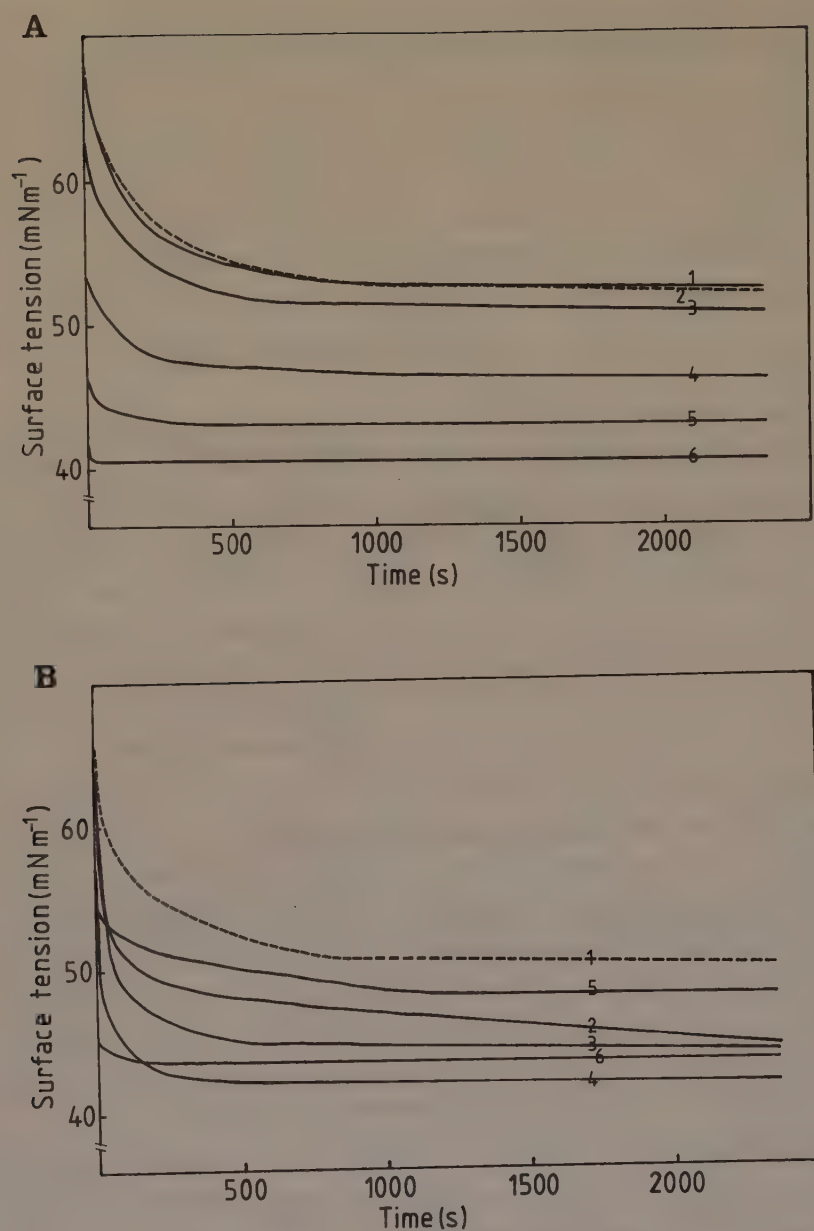


Fig. 12. Time-dependence of surface tension for solutions of ovalbumin in mixture with (A) monacaproin at pH 5.6 and (B) HPC at pH 4.0. (Scatter of measuring points as in Fig. 8). Protein concentration was kept constant at 0.9 g/l. Monacaproin concentration was 10^{-1} (2), $10^{0.6}$ (3), $10^{1.2}$ (4), $10^{1.5}$ (5) and $10^{1.7}$ (6) mM. HPC concentration was $10^{-2.6}$ (2), $10^{-1.8}$ (3), $10^{-1.4}$ (4), 10^0 (5) and $10^{0.4}$ (6) mM. Surface tension decay for pure ovalbumin is shown by the broken lines (1).

The time-dependence of surface tension decrease is more complex for the ovalbumin-HPC mixture, compared to the ovalbumin-monocaproin mixture. With HPC at concentrations below the cmc, mixed surface films appear to form. The equilibrium surface tension, as well as the surface tension decay at short times, are influenced by the presence of HPC at very low concentrations. At the lowest HPC concentrations investigated (1 to 10 μM), assuming a diffusion coefficient of $50 \cdot 10^{-11} \text{ m}^2 \cdot \text{s}^{-1}$, a diffusion time of minutes is to be expected. Thus, the increased rate in surface tension reduction cannot thus be explained by a faster diffusion of HPC, compared to the protein. Besides, an additional contribution to the electrical potential barrier to adsorption from increased net charge in the surface would result in a lower adsorption rate, as has been shown for pure protein (cf. Blank et al., 1975). The increased rate of surface tension reduction occurs at all mixing ratios examined, and the synergism noted in the surface tension reduction at near equilibrium conditions is thus pronounced already at short times.

Time-dependence of surface tension lowering also occurs in the time interval corresponding to penetration and reorientation of protein segments in the surface film (Fig. 8). Since surface tension reduction of HPC also shows time-dependence in this time interval, an analysis of these data is not meaningful. At the cmc where the marked minima occur in the equilibrium isotherms for the binary system, the kinetic curves are indistinguishable from the curves for pure HPC at intermediate concentrations, showing that HPC dominates in the surface layer. It is generally known that amphiphiles at sufficiently high concentration can replace protein in the interface film of protein stabilized emulsions, and thereby decrease emulsion stability (cf. Mulder and Walstra, 1974; Oortwijn and Walstra, 1979).

4.3 Enzymatic Activity

Catalytic activity of a protein is a sensitive indicator of disturbances in the native conformation. Enzymes generally have activity optimum at elevated temperature, but since inactivation processes also are forced at increasing temperature, decreased operational life-time will result. This is the main restriction for the use of enzymes in technological applications (Klibanov, 1983). Beside temperature, the sensitivity to pH and co-solutes are other limiting factors.

Selective response of enzymes to low molecular weight amphiphiles has been implied as a control mechanism for the composition and function of living cells (Tortora et al., 1978; Treves et al., 1984). Both naturally occurring amphiphiles (i.e. free fatty acids) and a synthetic one (i.e. SDS) have been shown to either inhibit, destabilize or stabilize various endogenous enzymes.

In papers IV and VI it is shown that amphiphiles can affect enzyme activity and stability through various mechanisms. Increasing solubility gives rise to increased activity, exemplified by the addition of cationic amphiphiles to lysozyme in paper IV, and further illustrated by the effect of HPC on lysozyme stability seen in Figure 13. Lysozyme solutions were heated at a heating rate of 1°C/minute. At appropriate temperatures samples were withdrawn, cooled and the residual activity was measured at 25°C (assay as in IV). The activity of pure lysozyme was reduced by 50% at heating to 95°C, while prolonged heating at 95°C for 25 minutes was required to reach the same inactivation in the lysozyme-HPC sample. The observed behaviour probably originates from solubility phenomena since

- 1) heat denaturation of lysozyme is highly reversible, and
- 2) deactivation is obvious at temperatures well below the denaturation temperature.

Consequently, the stability against the thermal deactivation is increased by the improved solubility induced by HPC. During prolonged heating at 95°C, besides aggregation, other secondary reactions occur, and the reversibility and hence the activity is lost.

The effect of various amphiphiles on enzyme stability reported in paper VI has been interpreted as due to alterations in protein structure, affecting its susceptibility to proteolytic digestion. In the absence of calcium ions, trypsin is known to show low stability against proteolytic cleavage, i.e. to autohydrolyse. The high susceptibility is due to a considerable amount of unordered secondary structure in the native conformation. Binding of Ca^{2+} -ions results in a more compact structure of trypsin (Lazdunski and Delaage, 1965), thereby increasing the stability against self-digestion.

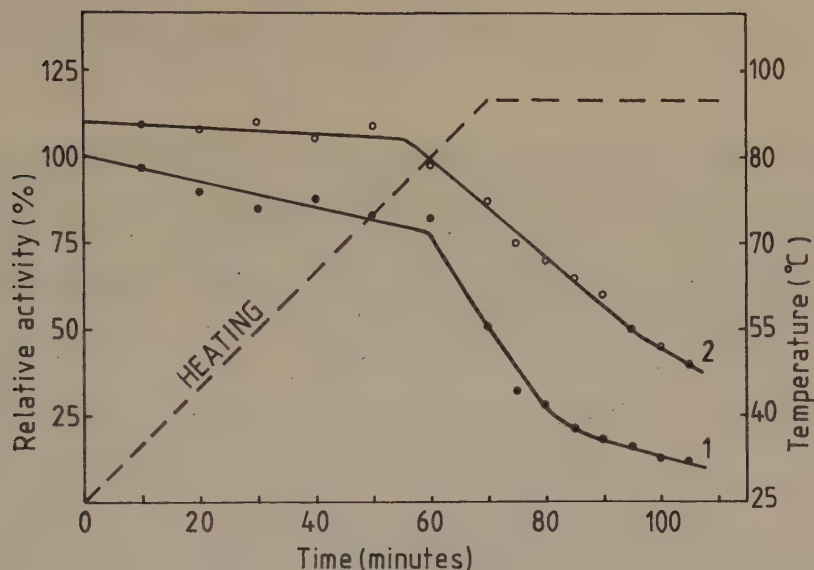


Fig. 13. Stability of pure lysozyme (1) and lysozyme with added HPC (0.4 g/g protein) (2) at pH 5, expressed as relative activity, with the activity of pure lysozyme at zero time as 100%. Protein concentration was 2.3 g/l. The samples were heated with a heating rate of 1°C/min from 20°C to 95°C (broken line, right-hand ordinate). The temperature was then kept constant at 95°C. Samples were withdrawn and residual activity was measured at 25°C (assay as in IV).

The cationic amphiphiles investigated (VI) have a similar effect on activity, stability and conformation of trypsin as Ca^{2+} - an effect which is achieved at low amphiphile-to-protein weight ratio (0.4 g/g trypsin). At higher weight ratios (~ 30 g/g trypsin) long-chain cationic amphiphiles have been reported to be competitive inhibitors for trypsin (Nakaya et al., 1976). Steric hindrance by a high amount of non-specific attached amphiphile molecules would probably resemble competitive inhibition.

The decrease in trypsin activity by anionic amphiphiles reported in paper VI is due to inactivation and not to inhibition. The trypsin conformation is destabilized, leading to an increased hydrolysis rate. A mechanism for the observed inactivation is proposed in VI. An interesting difference regarding the action of the fatty acid salts on trypsin was noted (VI). Hexadecanoate has a destabilizing effect on trypsin, whereas dodecanoate has a stabilizing effect. Tortora et al. (1978) have proposed a deactivating effect of long-chain fatty acids as a regulating factor *in-vivo* for

certain enzymes. The differences between the soaps observed here might be taken as evidence for such a regulation of trypsin activity in living systems.

The possibility to influence the unfolding path of a protein by an ionic surfactant, in such a way that the refolding process is facilitated, has been discussed (cf. III and paragraph 4.1.1). If this can be applied to an enzyme, with preserved activity at operational conditions, the increased reversibility would result in improved stability.

5. SUMMARY

Interactions between globular proteins and water insoluble as well as water soluble polar lipids have been studied.

The composite lyotropic mesophases formed with proteins and monoacylglycerols have been examined by low-angle X-ray diffraction, and the main features of the ternary aqueous lysozyme-monoolein phase diagram is given. Thermally induced transitions were followed by differential scanning calorimetry (DSC). It is concluded that native globular proteins can be accommodated in the water compartments of the cubic, reversed hexagonal and inversed micellar phases of aqueous monoacylglycerols. Technological and physiological relevance of the formed phases are outlined.

The binary aqueous monoolein phase diagram was reinvestigated - two cubic phases of bilayer structure exist adjacently in this system. The lipid bilayer forms an infinite periodic minimal surface in both cubic structures. Evidence is given for the transition of the body-centered cubic structure of the gyroid type (G) at low water content, to the primitive cubic structure of diamond type (D) with increased water content and/or temperature. The transition occurs without change in curvature (near energy-free) and is described by the Bonnet transformation. Attention is drawn to the importance of this transition for formation of the composite protein-monoacylglycerol-water cubic phase.

Protein structure, and thereby functions and properties, is influenced in different ways by water soluble polar lipids. In dilute aqueous medium, the effect of surfactants on protein conformation was studied by circular dichroism, and the degree of unfolding of the proteins was measured by enzymatic activity. Contrary to long-chain anionic surfactants, cationic surfactants do not generally cause denaturation of the proteins. The response of the formed complexes to heat was followed by DSC and specific optical rotation. Low concentrations of cationic surfactants were found to prevent thermal aggregation. At concentrations close to the cmc, also the thermal denaturation process is reduced. Increased reversibility of the thermal denaturation of proteins is reported. This can be achieved without disturbing native qualities at predenaturation temperatures. The interplay between attractive and repulsive forces is important for protein conformation in the formed complexes.

Surface tension at the air-water interface of globular proteins in solution with surfactants has been studied by the drop-volume method. Depending on protein-surfactant interaction, different patterns are seen:

1. A nonionic surfactant (monocaproin) drives the protein out of the surface at sufficiently high concentrations. No interaction, either in bulk solution or at the surface, takes place.
2. Specific binding of sodium dodecylsulphate to serum albumin does not influence the surface tension of the protein.
3. A synergistic effect was found for ovalbumin in ionic surfactants of low concentration, indicating interaction in the surface film.
4. Co-operative binding of ionic surfactants to the proteins in bulk solution gives rise to plateau regions in the surface tension reduction isotherms.
5. Hexadecylpyridinium chloride at concentration close to the cmc forms a hydrophilic complex with ovalbumin, which results in a minimum in the surface tension reduction isotherm.

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I

A CUBIC STRUCTURE CONSISTING OF A LIPID BILAYER FORMING AN
INFINITE PERIODIC MINIMUM SURFACE OF THE GYROID TYPE IN
THE GLYCEROLMONOOLEAT-WATER SYSTEM

by

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A cubic structure consisting of a lipid bilayer forming an infinite periodic minimum surface of the gyroid type in the glycerolmonooleat-water system

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Abstract. It is known that the monoolein-water system exhibits one cubic phase with a body-centered lattice at low water content and at higher water content another cubic phase, with a primitive lattice. Evidence is given for a structure of the body-centered phase with the lipid bilayer forming an infinite periodic minimal surface of the gyroid type. The transition from this structure to the primitive cubic structure with a lipid bilayer forming the diamond type of infinite periodic minimal surface, as proposed by Longley and McIntosh, can take place without change in curvature of the lipid bilayer. The transformation between these two minimal surfaces is described. The lattice parameters of the two cubic phases, the transition interval and an extremely low transition enthalpy are features consistent with such a transformation, which occur without change in curvature.

Introduction

A cubic structure in monoglycerides based on a lipid bilayer has been proposed by Lindblom et al. (1979). One cubic phase with a primitive lattice (Longley and McIntosh, 1983) and another with a body-centered lattice (Larsson, 1983) have been observed in the binary system, monoolein-water. Both structures have been proposed to be *infinite periodic minimal surfaces* (IPMS), with space group $Pn3m$ for the primitive lattice (Longley and McIntosh, 1983) and space group $Im3m$ or $Ia3d$ for the body centered lattice. These space groups correspond to the three fundamental cubic IPMS: the diamond type (D), the gyroid type (G) and Schwarz' surface (P), respectively

(cf. Schoen, 1970). The structure is thus formed by an infinite lipid bilayer, with the gap between the methyl end groups (the center of the bilayer) arranged according to the actual IPMS. In this way the lipid bilayer separates two continuous water net-works without connections.

In the present paper we will give evidence for a G-structure and a transition $G \rightarrow D$ with increasing amount of water and with increasing temperature in the binary system glycerolmonooleate-water. Furthermore, the phase diagram monoolein-water is given and the nature of this phase transition is discussed.

Discussion

The transition between a cubic IPMS of the gyroid type to the diamond type, $G \rightarrow D$, will first be considered from a theoretical point of view. Periodic minimal surfaces are best described in a transformed space, which is related to real space by two conformal mappings. The surface S_1 is first mapped into its spherical image, which is then mapped onto a complex plane via a stereographic projection. The surface is described in the complex plane by a two-dimensional function $R(\omega)$, where

$$\omega = \sigma + i\tau$$

The Cartesian dimensions of a point $P(x, y, z)$ on a minimal surface are related to the coordinates of its mapped point $P''(\sigma, \tau)$ by:

$$\begin{aligned} x &= Re \int_{\omega_0}^{\omega} (1 - \omega'^2) R(\omega') d\omega' + x_0 \\ y &= -Im \int_{\omega_0}^{\omega} (1 + \omega'^2) R(\omega') d\omega' + y_0 \\ z &= Re \int_{\omega_0}^{\omega} 2\omega' R(\omega') d\omega' + z_0. \end{aligned} \quad (1)$$

Re and Im refer to the real and imaginary, respectively. In terms of differential geometry, $R(\omega)$ completely determines the surface. O. Bonnet discovered 1853 that certain surfaces, S_1 , can be transformed into others, S_2 , with invariant intrinsic properties (for example curvature), which corresponds to bending without stretching.

$$S_1(R_1(\omega)) \rightarrow S_2(R_2(\omega)) = e^{i\theta}(R_1(\omega)),$$

where θ is the rotation of the doubly transformed surface about the normal to the complex plane and designates the extent of the transformation.

Such surfaces are called associate and θ is the degree of association. The cubic P- and D-surfaces are a special case, they can be transformed into each other by $\theta = \frac{\pi}{2}$, and are called adjoint.

Schoen (1970) discovered experimentally that a body-centered periodic minimal surface, the gyroid, with space group $I4_132$, could be obtained intermediate between the D- and P-surface by bending, with an angle of association of 38.015° with respect to the former.

A set of adjoint coordinates for the P- and D-surfaces were obtained by numerically solving the elliptic integrals from the parametrisation given in eq. (1), with:

$$R(\omega) = \frac{1}{\sqrt{1 - 14\omega^4 + \omega^8}}.$$

The gyroid boundaries were calculated using the Bonnet transformation of $\theta = 38.015$. A brass form was machined with these boundaries and used as a mould to produce plastic units to build the gyroid surfaces. (Hyde et al., 1984). By using minimal surfaces in the description of crystal structures, we proposed that the Gaussian curvature (as obtained from minimal surfaces or parallel surfaces) is proportional to bond strength. This was supported by simple explanations of the mechanism of zeolitic absorptions of gases, and of the catalytic and zeolitic cracking of molecules (Andersson et al., 1984), as well as enable us to describe the very fast martensite transition as a Bonnet transformation. Since the Bonnet transformation occurs with no change in curvature, phase transitions between phases described with the three surfaces discussed here should occur without heat exchange. They are all Bonnet related, i.e. they can be obtained from each other by bending with constant Gaussian curvature. Some interesting simple relations exist between the three surfaces. The metric relations between repetition units are for D, G and P:

1.00 : 1.11 : 1.28.

The G and D surfaces split space so that the volume relationships are for P, D and G (calculated from Schoen's surface/volume ratio):

1.07 : 1.022 and 1.00.

The symmetry of the lipid bilayer centered at the IPMS means that the symmetry of the lipid-water cubic phase is higher than that of the IPMS. Thus the space group of the gyroid $I4_132$ corresponds to space group $Ia3d$ of the whole structure.

Even if the X-ray data of the body-centered lattice showed extinctions which were in agreement with space group $Ia3d$, the choice of space groups has previously been kept open, due to the small number of observed reflexions (Larsson, 1983). It is proposed here, on the basis of evidence given below, that the body-centered cubic phase in the monoolein water system is an IPMS of the G-type. The methyl end group gap in the center of the lipid bilayer forms the actual IPMS, and the water channel systems form two intersection free networks on each side. The structure of the G-surface is shown in Fig. 1.

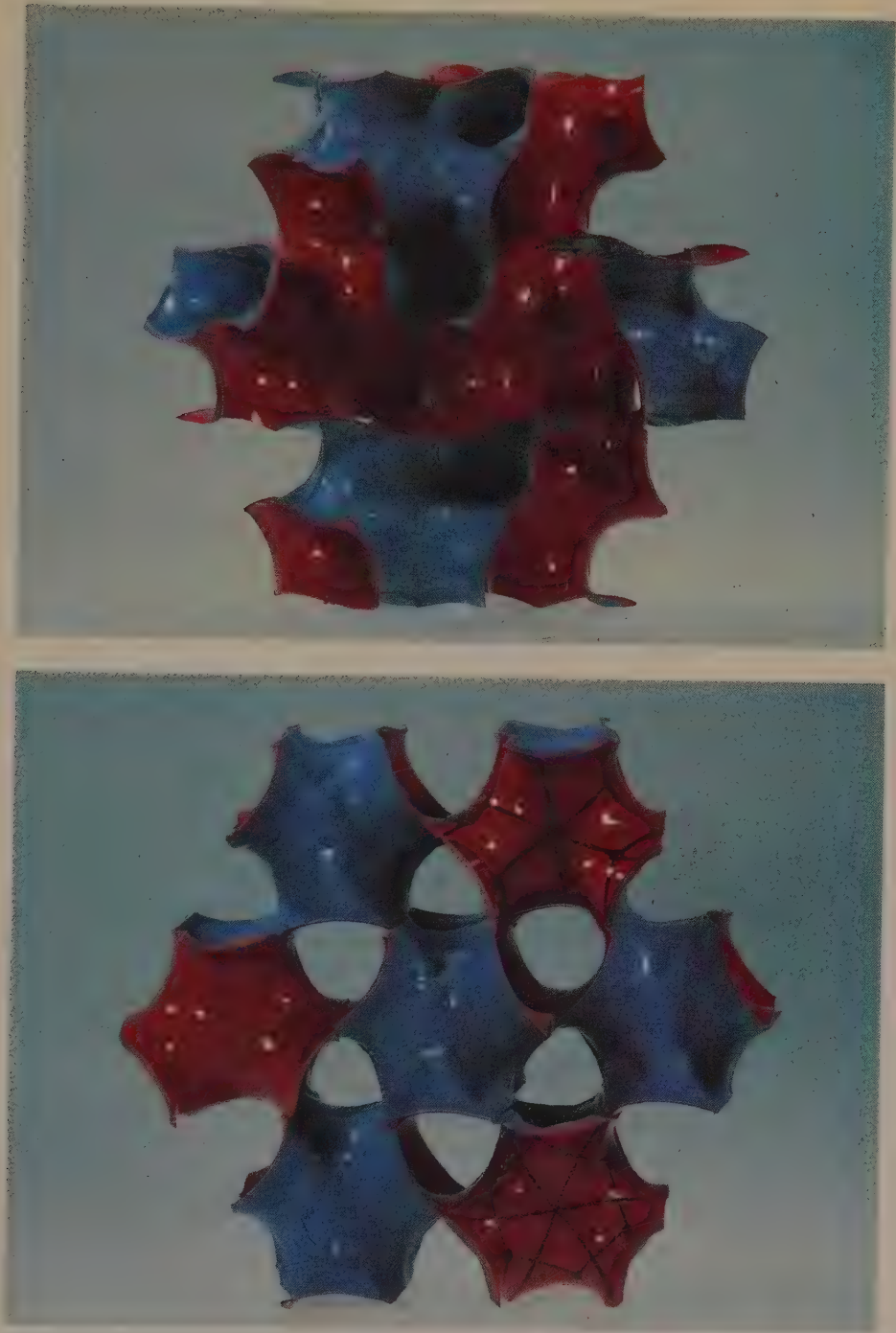


Fig. 1. The gyroid surface seen in two different directions with the two sides differently coloured

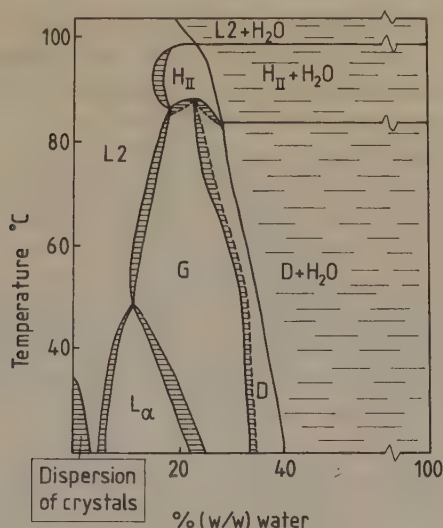


Fig. 2. The phase diagram of monoolein-H₂O. The cubic phases are denoted G (the gyroid type) and D (the diamond type). Other liquid crystalline phases are indexed L_α (the lamellar phase), H_{II} (the reversed hexagonal phase) and L₂ (the inversed micellar phase). The two-phase region dividing the cubic phases has been identified in the light microscope as a reduction of transparency, followed by an increase of transparency at the high temperature side of the two-phase region. These optical changes in relation to the two-phase region were confirmed by X-ray diffraction patterns. The later phase boundary is more uncertain (which is indicated in the diagram by a broken line)

The possibility that a phase transition can occur between two lipid bilayer structures without any change in curvature, as discussed above, means that the corresponding phases can be closely related from an energetic point of view. The phase transition enthalpies at the transition from the cubic to the hexagonal (H_{II}) phase in the monoolein-water system is about 1 kJ/mole. Phase transition enthalpies between other phases of lipid-crystalline character as well as transitions between liquid crystalline phases and liquid (L1 or L2) phases are known in numerous lipid systems, and they are also of the same order of magnitude. DSC-studies of the transition between the two cubic phases in the monoolein-water system, however, have not given detectable enthalpy values. From the accuracy of our experiment we can therefore conclude that the transition G → D exhibits an enthalpy less than about 0.01 kJ/mole. The fact that this phase transition is energetically so different from lipid phase transitions observed earlier is taken as an indication of a transformation of the bilayer structure without change in curvature, as described above.

The transition G → D is hard to observe visually. We have found that reduced degree of transparency in the two-phase region G → D can be used in

order to determine the phase boundaries. The phase diagram of the monoolein-water system is shown in Fig. 2. The appearance of the phase diagram is consistent with the $G \rightarrow D$ transformation taking place without change in curvature, as will be shown below.

General mechanisms behind phase transitions in lipid-water systems have been proposed by Israelachvili et al. (1976). Increase of temperature and increase of the water content result in an increased disorder from the polar group towards the end of the hydrocarbon chain. This is equivalent to a tendency for increasing the wedge-shape of the molecule, and the common transition from the lamellar phase (L_α) to the reversed hexagonal phase (H_{II}) upon heating can be explained in this way. From the phase diagram in Fig. 2 it can be seen that increase of the temperature as well as increase of the water content results in the phase transition $G \rightarrow D$. The water swelling of the G-phase results in a reduced curvature of the IPMS, and therefore in a decreased ratio between the molecular cross-section area at the methyl end group and the area at the polar head. In other words, the wedge-shape of the molecule is decreased during the swelling. When the temperature is increased, the volume occupied by the water increases, resulting in the same effect on the wedge-shape of the molecules. As the changes by heating and by swelling work against the normal tendency of increasing disorder towards the chain tail, it should be expected that heating as well as water swelling builds up strain, which ultimately results in a phase transition. The $G \rightarrow D$ transition described above can take place without change in the curvature of the lipid bilayer with an increase in volume per unit area of 2.2%. The effects on the molecular wedge-shape by heating and water swelling can thus be diminished by the transition $G \rightarrow D$.

The transition from the G-phase at 20 °C starts at about 33.5% (w/w) of water. According to the very small transition enthalpy it should be expected that the curvature of the lipid bilayer remains unchanged. The partial specific volumes of the lipid and water regions are about the same (cf. Lindblom et al., 1979), and it is therefore possible to calculate the water content of the co-existing D-phase. Thus a D-phase with 35.1% (w/w) of water has the same bilayer curvature as a G-phase with 33.5% (w/w) of water, which is consistent with observed boundaries (Fig. 2).

X-ray data of the G-phase have been reported earlier (Lindblom et al., 1979 and Larsson, 1983). The extrapolated values of the unit cell axes of the G-phase and the D-phase to the phase boundaries for co-existence at room temperature are 140.8 Å and 84.5 Å, respectively. The ratio between these axes 1.66 is close to the calculated ratio of $1.11 \cdot \sqrt{2} = 1.60$, which is obtained theoretically by the transformation $G \rightarrow D$ without change in curvature. The cross-section area per molecule in bilayer phases (L_α) of monoglycerides is about 30 Å², and the calculated value of the G-surface is 30.7 Å²/molecule at room temperature and the limit of swelling. These relations in geometry

between the G- and D-lattices as well as the molecular cross-section areas provide further evidence for the proposed structures.

It should be pointed out that the other body-centered alternative, the Schwarz' P-surface, is not consistent with the observed relationship in lattice parameters or with expected volume changes at the transition to the D-lattice.

The possibility that other cubic phases described in various lipid-water systems consist of IPMS should be considered. The liquid-like structure of the lipid bilayer requires a structure where all molecules have equivalent packing conditions. There is a pronounced tendency for formation of a bilayer structure in aqueous phases of lipid molecules, due to the amphiphilic nature of the molecules, and any intersection of bilayers would result in irregularities in the molecular packing. The requirement of a cubic structure consisting of an intersection-free infinite layer can hardly be fulfilled by any other type of structure.

Luzzati and coworkers (1968) have suggested that networks formed by rods of finite length represent a general structure of cubic lipid phases on the basis of X-ray diffraction data corresponding to space group *Ia* $3d$. The rods in this model are tangential axes to the helical space on each side of the IPMS of the gyroid.

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II

A CUBIC PROTEIN-MONOOLEIN-WATER PHASE

by

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BBA 71581

A CUBIC PROTEIN-MONOOLEIN-WATER PHASE

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Key words: Cubic phase; Monoacylglycerol; Lipid-protein-water; Lysozyme; X-ray diffraction; Differential scanning calorimetry

A cubic monoacylglycerol-protein-water phase has been identified by low-angle X-ray diffraction, and the main features of the ternary phase diagram monoolein/lysozyme/water are presented. The thermal stability of the protein in the lipid-protein cubic phase has been examined by differential scanning calorimetry. According to the physical properties of the phase it is proposed that the protein molecules are located in the water medium, i.e. in the water channel systems of the cubic structure earlier suggested. The ability of various proteins to form this cubic phase has been studied, and it was found that the formation of this phase is favoured by an isoelectric point (pI) far from pH 7 in a salt-free solution, thus by high electrostatic repulsive forces.

Introduction

The interaction of proteins with lipids, with special regard to the formation of aqueous liquid-crystalline phases, has been studied in various systems (cf. Refs. 1 and 2). One general conclusion from these results has been that mesomorphic lipid-protein-water phases are formed only when there is a possibility for ionic interaction between lipid and protein molecules [3]. The cubic phase described in the present paper is in this respect different from the phases earlier observed. As reported below proteins with a wide variation in size (about 14–150 kDa) are able to form a cubic phase with monoolein. The dimensions of the lattice depend on the proportion of protein and water present. The formation of this lipid-protein phase is discussed in relation to the cubic monoolein-water structure earlier proposed, with a continuous lipid bilayer which separates two water channel systems [4].

Little is known from earlier studies about the protein structure in such lipid-protein liquid-crystalline phases.

In a Raman spectroscopy study of insulin-phospholipid-water phases [5], it was reported that the protein kept its native structure provided the lipid chains were below a certain length. The present work has been focused on the thermal stability of the protein structure in a cubic lipid-protein phase.

Materials

The 1-monoolein used (more than 99% pure, lot No. 110F-0481) was from Sigma Chemical Co. Lysozyme (L-6876, lot No. 57C-8025), α -lactalbumin (L-4379, lot No. 50F-8105), soybean trypsin inhibitor (T-9003, lot No. 68B-0560), myoglobin (M-0380, lot No. 85C-0138), pepsin (P-7012, lot No. 16C-7201), bovine serum albumin (A-7511, lot No. 16C-7201), conalbumin (C-0755, lot No. 39C-8000), and glucose oxidase (G-2133, lot No. 66C-0201) were all purchased from Sigma Chemical Co. Lysozyme, myoglobin and pepsin were dialyzed against distilled water for 24 h followed by freeze-drying to constant weight.

Methods

Preparation of samples. Samples were prepared at 40°C by slow addition of the aqueous protein solution (prepared in double distilled water) to the liquid monoacylglycerol. The samples were then allowed to equilibrate at room temperature for a maximum of 24 h (or until no change in the polarizing microscope could be detected).

A one-phase region of the cubic phase is simpler to identify than other mesophases. This phase is completely transparent, very viscous and optically isotropic. Still it was checked by centrifugation ($8000 \times g$ during 48 h) that no phase separation can be achieved especially that no excess protein solution was present.

X-ray diffraction. The samples were identified by a Guinier type of low-angle camera according to Luzzati and a Kiessig type of point-focus camera as described earlier [6].

Microscopy. For microscopic examination of the samples an Olympus Vanox polarizing microscope was used. A heating gradient, ranging from 25°C to 110°C was achieved by a Mettler FP52 heating table, using a heating rate of 10 K/min.

Differential scanning calorimetry. Calorimetric measurements were performed in a Perkin-Elmer DSC-2C calibrated according to Ref. 7. Samples (approx. 5 mg) were weighed into Perkin-Elmer standard volatile sample aluminium pans (part No. 219-0062), using an empty pan as reference.

Results

The phase diagram

Fig. 1 shows the main features of the ternary phase diagram monoolein/lysozyme/water at 40°C. The phases were identified in the polarizing microscope and by X-ray diffraction.

A limiting factor for the lysozyme concentration in this ternary system is the high viscosity of the protein solution above about 45% (w/w). Due to this experimental difficulty we have only information on the phase equilibria in the concentration region below 45% (w/w) lysozyme in water (see Fig. 1). Furthermore, the phase equilibria above a lipid concentration of 80% (w/w) were extremely slow, and we have therefore omitted also this region of the phase diagram.

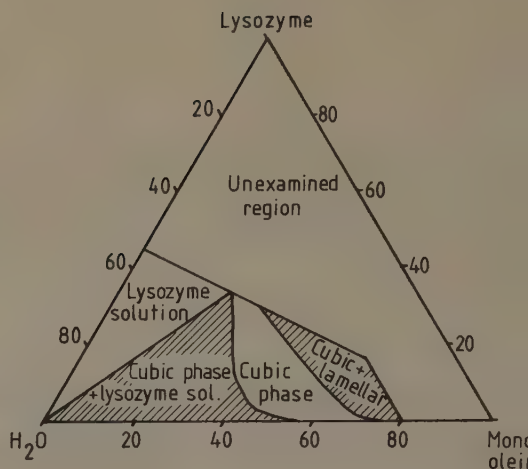


Fig. 1. Ternary phase diagram of the system monoolein/lysozyme/water at 40°C.

At 40°C the binary system monoolein/water forms a 'viscous isotropic' phase [8] at water concentrations from 20 to 40% (w/w). This structure has been characterized by low-angle X-ray diffraction [4,6] to be a body-centered cubic structure. This cubic phase has been proposed to be composed of bilayer units forming a continuous three-dimensional network, which separates two identical water-channel systems. The same type of cubic phase is formed in the ternary system lysozyme/mo-
noolein/water according to X-ray diffraction data. As can be seen from the phase diagram (Fig. 1), the phase boundaries for the cubic phase are shifted towards higher water content as compared to the binary monoolein/water system, already at low lysozyme concentrations.

X-ray diffraction

X-ray diffraction data on the cubic phase reveal, as a result of the raised water content, an increase of the lattice dimensions when lysozyme is present. Another interesting effect due to the protein is the temperature fall-off of the reflection intensities, which is reduced so that many more reflections can be observed. An illustrative example of such a phase exhibiting 28 reflections is given in Table I.

Microscopy

Thermal phase transitions were followed in the

TABLE I

X-RAY DIFFRACTION DATA OF THE CUBIC PHASE OF MONOLEIN/LYSOZYME/WATER (31.6:34.2:34.2, w/w)

All diffraction lines fit into a body-centered cubic lattice ($a_0 = 235$ Å) but only the low-order reflections can be indexed unambiguously. For lower lysozyme concentrations the lattice is smaller, e.g. the composition monoolein/lysozyme/water (56.0:6.6:37.4, w/w) gives $a_0 = 215$ Å. The values of a_0 for monoolein-water cubic phases at different water contents are given by Lindblom et al. [6].

d (Å)	hkl	a_0	I^a	d (Å) (contin.)	I^a
96.6	211	236.6	vs	17.33	w
82.97	220	234.7	s	16.14	w
68.49	222	237.3	w	15.03	w
54.62	411, 330	231.7	w	14.24	vw
41.09	440	232.4	m	13.36	w
36.26	541	235.0	m	12.32	w
32.77	640	234.2	w	11.39	w
29.02	Mean value		w	10.74	w
28.02		234.6	w	10.22	w
27.08			w	9.79	vw
23.45			vw	9.40	vw
21.50			w	9.13	vw
20.81			w	9.05	vw
19.44			w	8.55	vw

^a I = intensity, using an intensity scale of vs = very strong, s = strong, m = medium, w = weak, vw = very weak.

polarizing microscope. The temperature induced transitions of the binary system monoolein/water have been reported by Lutton [8]. In the excess of water region (above 40% (w/w) water) there is a transition at heating of the cubic phase at 90°C to a hexagonal phase. Further heating to above 100°C results in a transition from the hexagonal phase to an isotropic fluid (L2-phase according to Ekwall's nomenclature [9]). The same phase transitions were found to take place also when the cubic monoolein/water phase contained protein. Thus, the hexagonal phase was found to appear at a temperature of 91–93°C, evident in the polarizing microscope from the characteristic texture of the sample. The transition into the L2-phase, evident from the disappearance of birefringence and a 'melting' of the sample, was observed at a temperature of about 96–102°C. The reversed behaviour was seen on cooling.

Differential scanning calorimetry

Fig. 2 shows a typical thermogram of a sample of the cubic phase containing lysozyme. Protein denaturation occurs at 64°C (T_D in Fig. 2), involving an enthalpy of denaturation of about 300 kJ/mol protein. Both the temperature and the enthalpy values are typical for the samples of the cubic phase containing lysozyme in various concentrations (ranging from 6% (w/w) lysozyme to 34% (w/w) lysozyme). That is, no difference related to protein concentration either in temperature or in denaturation enthalpy can be seen. The values obtained are in good agreement with results of Fujita and Noda [10] from studies on lysozyme at low and intermediate water content. Thus in the lysozyme concentration range examined here, lysozyme in the cubic phase shows native qualities as regard temperature and enthalpy.

Lysozyme is known to be reversible on heat denaturation [11]. It was found that the reversibility of lysozyme in the cubic phase, however, depends on kinetic parameters, i.e. heating and cooling rate, and also on the maximum temperature the sample has been exposed to. According to DSC-measurements at reheating of the samples, reversibility ranges from around 90% (high and intermediate heating/cooling rates, the sample not exposed to temperatures above 100°C) to 0% (slow

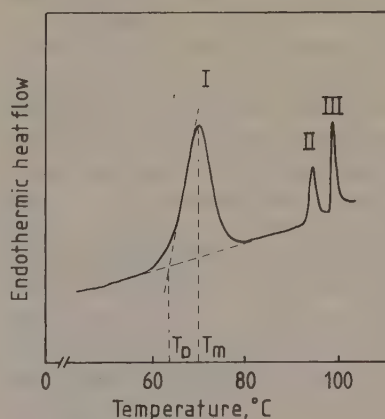


Fig. 2. Thermogram of a cubic phase sample with the composition monoolein/lysozyme/water (35.6:25.1:39.3, w/w). Peak I corresponds to denaturation of the protein, peak II corresponds to the phase transition cubic $\rightarrow$ hexagonal and peak III corresponds to the phase transition hexagonal $\rightarrow$ L2 (inverted micellar phase).

heating/cooling rates, maximum temperature above 100°C). Thus, the degree of reversibility seems to be dependent on the time-temperature profile above the hexagonal to L2-phase transition, which involves a 'melted' phase. This transition occurs, dependent on the composition of the samples, between 96–102°C, i.e. near the boiling point of the water.

The phase transition identified by the polarizing microscope can also be seen in the thermograms (cf. Fig. 2). The transition from cubic phase to hexagonal phase occurs as an endothermic peak at 96°C, and on further heating there is a second endothermic peak revealing the transition into the L2-phase at 99°C. Both these transitions involve enthalpies in the magnitude of 0.75 kJ/mol monoolein.

Other proteins forming the cubic phase with monoolein and water

It has earlier been reported that a very hydrophobic wheat protein fraction, gliadin, could be solubilized into the cubic monoolein-water phase [12]. Thus 10% (w/w) of A gliadin dispersed in 90% (w/w) monoolein, formed the cubic phase when water was added. Gulik-Krzywicki et al. [13] in a recent freeze-etching electron microscopy study of the cubic structure, has found that an aqueous cytochrome *c* solution can form this cubic phase with sunflower oil monoacylglycerol.

To examine whether the effect of lysozyme on the cubic monoolein-water phase is a phenomenon general to hydrophilic proteins, a few other globular proteins were examined. The composition of these new protein samples was chosen to be approx. 40% (w/w) monoolein/18% (w/w) protein/42% (w/w) water. The proteins studied were α -lactalbumin (M_r 14 200, pI 4.2–4.5) [14], soybean trypsin inhibitor (STI) (M_r 20 100, pI 4.0) [15], myoglobin (M_r 17 000, pI 7.0) [16], pepsin (M_r 35 000, pI 1.0) [16], bovine serum albumin (BSA) (M_r 67 000, pI 4.5–5) [17,18], conalbumin (M_r 77 000, pI 6.5–6.8) [19,20], and glucose oxidase (M_r 150 000, pI 4.2) [21].

α -Lactalbumin, bovine serum albumin and pepsin resemble lysozyme when they form the cubic phase. They give after equilibration for 24 h a viscous transparent phase, which is optically isotropic, and shows the same characteristics as the cubic monoolein-water phase. Also myoglobin

forms the cubic phase together with monoolein. The behaviour is different, however, as a prolonged equilibration time was needed (for the weight ratio 7 days) in order to obtain a homogeneous cubic phase.

α -Lactalbumin and lysozyme are small proteins similar in size and closely related regarding the amino acid composition and structure [22], which can explain the resemblance in their phase behaviour. Bovine serum albumin, on the other hand, is a much larger protein, which indicates that the geometry of the cubic lattice is flexible, allowing hydrophilic proteins of different sizes to be introduced.

As lysozyme and pepsin carry a high net charge (pI near 11 and 1, respectively), whereas myoglobin has an pI of 7, electrostatic repulsion forces might be of importance in the formation of the ternary cubic phase. These three proteins had been desalted before the addition to the monoacylglycerol, i.e. the shielding effect of salt present in the aqueous protein solutions has been diminished. If these proteins are added prior to desalting the phase behaviour is quite different. The myoglobin/monoolein sample gives after equilibration a cubic monoolein-water phase in contact with excess protein, whereas pepsin and lysozyme enter the cubic phase, but at an extremely slow rate.

From this it might be concluded that high apparent net charge repulsion between the protein molecules favours the formation of the cubic monoacylglycerol-protein-water phase. The other proteins examined behave in a similar way, thus even the largest protein used in this investigation, glucose oxidase (M_r 150 000), can form the cubic phase.

Discussion

The cubic protein-monoacylglycerol-water phase described above exhibits similar intensity distribution of the X-ray diffraction pattern as the monoolein-water phase [6]. Thus, there are no reasons to suggest any structural differences in relation to the bilayer model proposed [4]. It can be assumed that the protein molecules are not attached to the lipid bilayer matrix but is located in the water channel systems according to the following evidence:

1. Lysozyme activity can be observed outside the cubic phase (a suspension of dried cells of *Micrococcus lysodeikticus* was added to the cubic phase containing lysozyme and enzymatic activity was observed in the suspension outside the cubic phase).

2. Diffusion of coloured proteins can be followed visually. It can thus be seen how proteins such as myoglobin move into the cubic monoacylglycerol-water phase from an outside protein solution or out from the cubic monoacylglycerol-protein-water phase to an outside water solution.

3. A cubic monoacylglycerol-lysozyme-water phase can be dried over vacuum until all the water is removed, and the same aqueous phase can then be reconstituted by water exposition with the protein still in its native state as measured by DSC.

4. The enthalpy and temperature of thermal denaturation are the same for the protein in the cubic phase and in aqueous solution.

The difference in behaviour of the hydrophilic proteins examined can possibly be explained by the difference in net charge. Electrostatic repulsion forces between the protein molecules might influence the formation of the cubic lattice. Proteins with isoelectric point (pI) far from pH 7, thus with high net charge in a water solution, seem to have a higher ability to increase the dimension of the cubic lattice than proteins with pI near 7. The effect of the salt concentration, i.e. the shielding of the electrostatic repulsion, is in accordance with this model. Thus, when the protein carries an apparent net charge, the formation of the cubic protein-monoacylglycerol-water phase is facilitated, and equilibration time required is shortened.

The cubic monoacylglycerol-water phase can be used to induce membrane fusion. Monoolein, for example, is a well-known fusogenic substance, and evidence for the formation of a cubic structure in connection with fusion has been reported [23]. A possible mechanism might be that a fragment of the cubic phase adheres to the actual membrane by a local joint between the bilayer of the cubic phase and the membrane bilayer. When the bilayers are joined, the lipid composition will tend to be equally distributed by the rapid lateral diffusion. If the cubic 'particle' constitutes a minor part of the total bilayer system, it should thus be expected to be solubilized in the membrane. If there also is a joint between the cubic fragment and another membrane, such a diffusion should lead to

fusion, and any protein in the cubic phase will also be involved in such a fusion process.

Acknowledgements

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III

PROTECTION OF OVALBUMIN AGAINST IRREVERSIBLE HEAT DENATURATION BY A CATIONIC AMPHIPHILE AT HIGH CONCENTRATION

by

B. Ericsson, P.-O. Hegg and K. Mårtensson

Protection of ovalbumin against irreversible heat denaturation by a cationic amphiphile at high concentration

B. ERICSSON*†, P.-O. HEGG* AND K. MÅRTENSSON‡

Summary

The interaction of the cationic amphiphile cetylpyridinium chloride (CPC) with the egg white protein ovalbumin, was studied at various temperatures and pH values. The influence of different concentrations of CPC on the thermal behaviour of the protein was followed by specific optical rotation measurements in the pH interval 3–9. No increase in optical rotation was found at any pH when CPC was added to ovalbumin at room temperature. When heated, a gradual increase in optical rotation was registered, which became smaller the higher the pH used. At molar ratios of CPC : ovalbumin of above 70 this increase was reversed on cooling. The reversibility of the thermal unfolding on cooling was not affected by a variation in protein concentration between 0.5 and 10%. Circular dichroism measurements before and after a heating–cooling cycle confirmed the results obtained on optical rotation. When CPC was exchanged against the anionic amphiphile, sodium dodecylsulphate (SDS), a denaturing effect was achieved at neutral and alkaline pH values already at room temperature. At the acidic side of the isoelectric point, however, there was no major effect of SDS. No thermal aggregation of ovalbumin occurred near the isoelectric point when CPC was present and complete resolubility was found after thermal treatment and drying.

Introduction

The understanding of how to influence functional properties of proteins in food is still poor. However, precipitation and gelling properties of proteins under a thermal treatment have been investigated in some detail (Hegg, 1978). It was found that thermal aggregation could be influenced by rather simple methods.

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An increased salt concentration strengthened aggregation, while a high or low pH usually had the opposite effect. Even an inhibition of thermal aggregation of proteins could be achieved by adding small amounts of the anionic amphiphile sodium dodecylsulphate to the protein solution. Unfortunately, however, this leads to a simultaneous denaturation of the protein structure, which possibly changes the functional properties of the protein in a following food production process. Modification of protein properties without obtaining significant losses in protein structure is thus especially important in food technology. As thermal treatments are so frequent in food processing, also a protection against protein denaturation in general would be of technological importance. This is particularly true in processes where a protein structure with native qualities is desired after a unit operation where denaturation may occur, e.g. a pasteurization process (Lineweaver *et al.*, 1967). Protection is also particularly important if enzymatic activity is desired during or after such a process.

In model studies of the interaction between proteins and amphiphiles mostly bovine serum albumin (BSA) and sodium dodecylsulphate (SDS) have been used. Bovine serum albumin (BSA) has been reported to bind anionic amphiphiles with high affinity (Steinhardt & Reynolds, 1969) and at the same time become stabilized against structural stress as, for instance, heat (Gumpen, Hegg & Martens, 1979). The only protein reported with amphiphile binding properties similar to that of BSA is β -lactoglobulin (Steinhardt & Reynolds, 1969; Hegg, 1980). With the exception of a few proteins, e.g. glucose oxidase, papain and pepsin, which show a high resistance against unfolding by SDS (Nelson, 1971), all other proteins examined so far seem to be structurally transformed by this amphiphile (Lapanje, 1978).

If a minimal effect on protein structure is desired as a result of binding of an amphiphile, SDS seems to be an inappropriate choice. The twelve carbon skeleton, which appears to induce a maximal unfolding of proteins (Tanford,

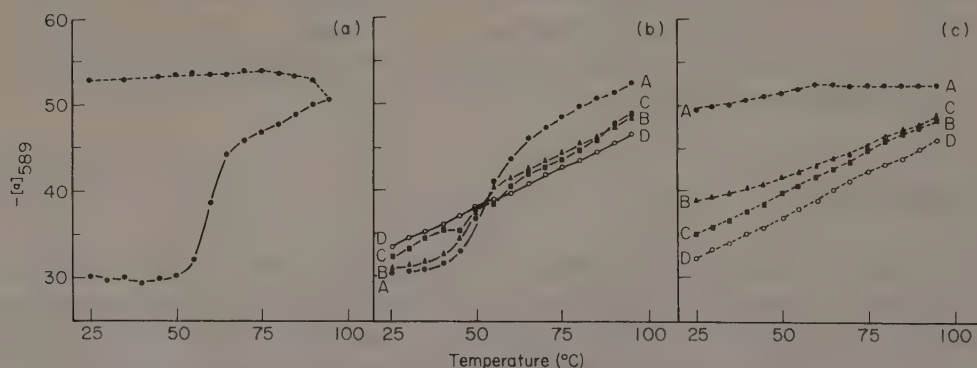


Figure 1. Specific optical rotation of ovalbumin and ovalbumin dissolved in cetylpyridinium chloride (CPC) at pH 3 as a function of temperature during heating and cooling. Protein concentration: 25 mg/ml. (a) Ovalbumin without CPC added. —, Heating (1°C/min); ---, cooling (1°C/min). (b) Heating of ovalbumin (1°C/min) in CPC of different concentrations. A, 8 mM CPC; B, 20 mM CPC; C, 40 mM CPC; D, 200 mM CPC. (c) Cooling of ovalbumin (1°C/min) in CPC. Concentrations as in (b).

1973; P.-O. Hegg, unpubl. data) combined with the sulphate head group, which intensifies the affinity to the protein more than most other head groups (Steinhardt & Reynolds, 1969), makes this amphiphile a strong denaturing agent. Thus the denaturing effect might be avoided if an amphiphile with a more apposite hydrophobic-hydrophilic balance is chosen. The interaction with such an amphiphile would probably result in a protein molecule with changed functional properties, but in addition there would also be a possibility to attain a protein structure less susceptible to external stress. The influence of amphiphiles other than SDS on protein structure is, however, poorly examined. This is particularly the case with the cationic ones.

The intention of the present work was to examine the effect of a cationic amphiphile on protein structure at different temperatures. As a model system cetylpyridinium chloride (CPC) and the egg white protein ovalbumin was used. The results obtained with CPC were compared with those obtained with SDS.

Materials and methods

Materials

Ovalbumin was prepared from hen's egg white according to Hegg (1979). No other egg white proteins could be detected in the protein preparation by SDS gel electrophoresis. Sodium dodecylsulphate (Lot No. L-5750) and cetylpyridinium chloride (Lot No. C-9002) were purchased from Sigma Chemical Co., U.S.A.

Methods

Optical rotation. The optical rotation measurements were carried out in a Perkin-Elmer 141 polarimeter at the wavelength of sodium-D-line (589 nm). The polarimeter cell (1.0 dm, 5 ml) was thermostated to temperatures within the interval 25 to 95°C at a heating and cooling rate of 1°C/min. This was obtained by coupling the polarimeter cell to a circulating water bath equipped with an electronic heat gradient control (Heto, Denmark, type 02 PH 623). Transition temperature is defined as the temperature at the midpoint of transition. Data are expressed as specific optical rotation with an accuracy of $\pm 0.5^\circ$.

Circular dichroism. Circular dichroism measurements were performed using a JASCO J-40 dichrograph in the wavelength range of 270–210 nm. Results are expressed as mean residue ellipticities (degree cm²/dmol), using the value 112 for the mean residue weight of ovalbumin. A protein concentration of 0.5 mg/ml and a cell width of 0.1 cm was used in these experiments. All measurements were made at 25°C. As ovalbumin does not give a significant dichroism signal in the near u.v. region, only the far u.v. region, yielding information about the backbone conformation of the protein, was examined.

Preparation of solutions. Protein solutions of twice the final concentration were prepared using distilled water. Equal volumes of amphiphile solutions,

also of twice the desired concentrations, were then added slowly and under stirring. In most experiments a final protein concentration of 25 mg/ml was used. After addition of amphiphile the solutions were adjusted to appropriate pH using 1.0 M HCl or NaOH. A precipitate formed above the isoelectric point of ovalbumin during pH adjustment of the ovalbumin-CPC solution. This precipitate dissolved upon stirring. The time required for resolution was dependent on the pH of the solution, but was typically 1 hr.

All solutions used for analysis were optically clear and freshly prepared.

Results and discussion

Effect of cetyl pyridinium chloride on the thermal denaturation of ovalbumin at pH 3

At pH 3 ovalbumin carries a positive net charge of about 25 (Hegg, 1978; Nakamura, Hirai & Takimori, 1980), and due to net charge repulsion it does not aggregate when heated to temperatures above the denaturation temperature so far from the isoelectric point (4.6–4.9) (Rhodes, Azari & Feeney, 1958; Cannan, Kibrick & Palmer, 1941). Precipitation below the isoelectric point with anionic amphiphiles and above the isoelectric point with cationic has earlier been reported (Hegg, 1979) at low concentrations of added amphiphile. This phenomenon is thus not to be expected when the cationic amphiphile CPC is added at pH 3. These two facts made pH 3 suitable for examining the effect of various amounts of CPC on the thermal denaturation of ovalbumin.

The specific optical rotation (OR) of ovalbumin during heating and cooling is shown in Fig. 1a. In the absence of amphiphile the heat denaturation process is fast with a transition point at 59°C. On cooling the OR value does not return to the initial level, but remains above that obtained at 95°C. This is indicative of an irreversible process of denaturation, and is in accordance with earlier reports on ovalbumin (Smith, 1964).

From Fig. 1b it is seen that addition of various concentrations of CPC does not significantly alter the specific optical rotation at 25°C. This indicates that no unfolding of the protein structure occurs at this temperature. When heated at low amphiphile:protein ratios the thermal stability of the protein becomes lowered. Thus, the transition point decreases from 59 to 55°C at 8 mM CPC (molar ratio CPC:ovalbumin of 14) and to 50°C at 20 mM (molar ratio of 36). At higher amphiphile concentrations the sharp transition is replaced by a gradual increase in specific rotation and no defined denaturation temperature can be detected. The final optical rotation value obtained at 95°C is also lowered by 8° compared to when CPC is absent. An increase in amphiphile concentration above 40 mM (molar ratio of 72) did not alter the optical rotation curve further.

The behaviour of ovalbumin on cooling at the different concentrations of CPC is given in Fig. 1c. At low CPC concentrations there is a slight decrease in the OR value as the temperature is decreased, but the major transition is not

reversed. However, for the two highest concentrations of amphiphile, the cooling process is the reverse of the heating process. Above 80% of the $\Delta[\alpha]_d$ is regained upon cooling suggesting that the heat-induced unfolding has become reversible.

Effect of pH

To examine the contribution of electrostatic forces for the formation of the protein–amphiphile complex, optical rotation measurements were carried out at increasing pH values.

The precipitate formed at and above the isoelectric point at low concentrations of added cationic amphiphile will redissolve at a sufficiently high concentration of amphiphile (Hegg, 1979). To be able to measure optical rotation of ovalbumin in CPC above the isoelectric point a concentration of 40 mM (corresponding to a molar ratio of amphiphile : protein of 72) was required. Notably, this concentration was also the requisite to obtain protection against irreversible heat denaturation at low pH (Fig. 1c).

Only a marginal increase in specific rotation was found at 25°C with increasing pH (Fig. 2), and the reversibility of the thermal denaturation process was completely maintained at all pH values examined. However, a gradual decrease in the slope of the curves was found with increasing pH when optical rotation was plotted as a function of temperature. This suggests that there is an electrostatic contribution to the protein–amphiphile interaction, and that complexes, which are progressively less heat susceptible, are formed when the negative charge on the protein increases. But since the interaction between ovalbumin and CPC is manifest already at pH 3, when both kinds of molecules are positively charged, hydrophobic interactions must also be of importance for the formation of the complex (Fig. 1b).

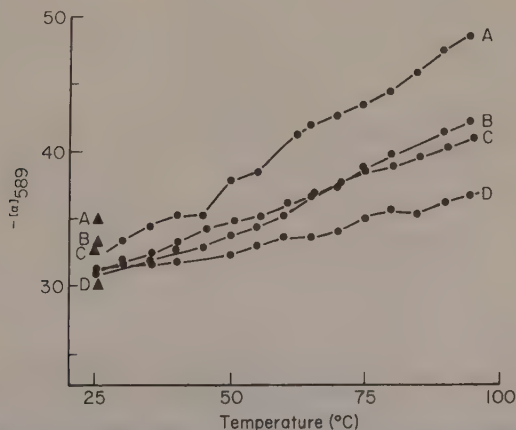


Figure 2. Specific optical rotation of ovalbumin (25 mg/ml) in 40 mM cetylpyridinium chloride (CPC) as a function of temperature at different pH values. A, pH 3; B, pH 5; C, pH 7; D, pH 9; ▲, Values after cooling.

Circular dichroism

It can be seen from the optical rotation curves (Figs 1 and 2) that ovalbumin in presence of CPC attains the same, or a very similar value of $-\left[\alpha\right]$ after heating and cooling as before heating. In order to compare the conformations corresponding to these values circular dichroism measurements of the two solutions were performed at pH 3 and the results are given in Fig. 3. At the wavelengths examined ovalbumin gave rise to a negative signal (Curve A), which diminished considerably after heating to 95°C (Curve B). The protein lost its ordered structure when denatured by heat and did not regain it on cooling. With CPC present the CD signal increased about 10% (Curve C). This small change might indicate that a slightly more dense complex is formed. The signal of the heat treated ovalbumin–CPC solution was identical to that obtained before heating (Curve D). These results show that the protein conformation is retained after the heating–cooling cycle, i.e. that CPC hampers the irreversible thermal unfolding of ovalbumin.

Effect of protein concentration

Ovalbumin normally becomes irreversibly denatured when heated. The amphiphile CPC has the ability not mainly to protect against heat denaturation but more to make the denaturation reversible. It is reasonable to assume that the reversibility of a denaturation process should be facilitated when the protein

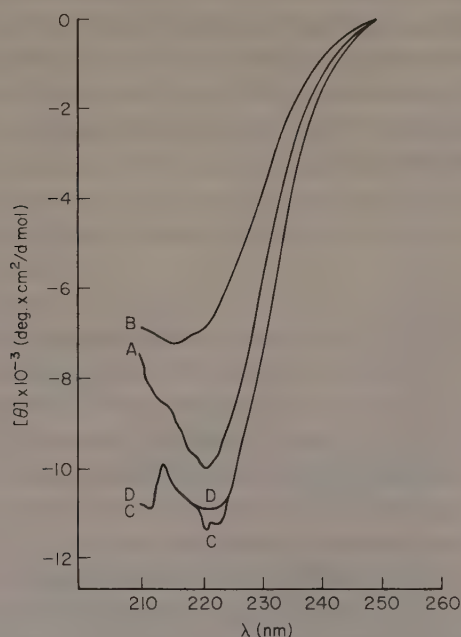


Figure 3. Circular dichroism of ovalbumin and ovalbumin dissolved in cetylpyridinium chloride (CPC) at pH 3. A, ovalbumin prior to heating; B, ovalbumin after heating to 95°C; C, ovalbumin in CPC (72 mol CPC/mol ovalbumin) prior to heating; D, the same as curve C after heating to 95°C.

concentration is decreased due to intermolecular interactions. To examine whether the reversibility of the heat denaturation process in the presence of CPC was influenced by protein concentration this was varied keeping the molar ratio of CPC to protein in (72 : 1) constant.

A protein concentration of 25 mg/ml has been used throughout this investigation. No influence on the reversibility was found when protein concentrations of 5 and 100 mg/ml were examined. However, when the protein concentration was increased a parallel displacement towards lower specific optical rotation values occurred.

Effect of SDS

Figure 4 shows the effect of 40 mM SDS on ovalbumin at pH 5, 7 and 9. At this concentration of SDS optical rotation measurements at pH 3 were not possible due to aggregation on heating. In contrast to CPC, the effect of SDS on ovalbumin seems to be extremely dependent on pH.

At pH values where ovalbumin carries a negative net charge, SDS has a strong influence on the optical rotation behaviour. Thus, the initial OR value at 25°C is increased by about 10 at pH 7 and 9, i.e. the protein conformation is altered already by the addition of SDS. Only a marginal further increase in optical rotation was registered when the temperature was raised to 95°C. Thus the protein conformation once formed at low temperature seems to be resistant to heat.

The effect of SDS on ovalbumin at and below the isoelectric point was completely different from that registered at pH 7 and 9. At pH 5 the OR curve

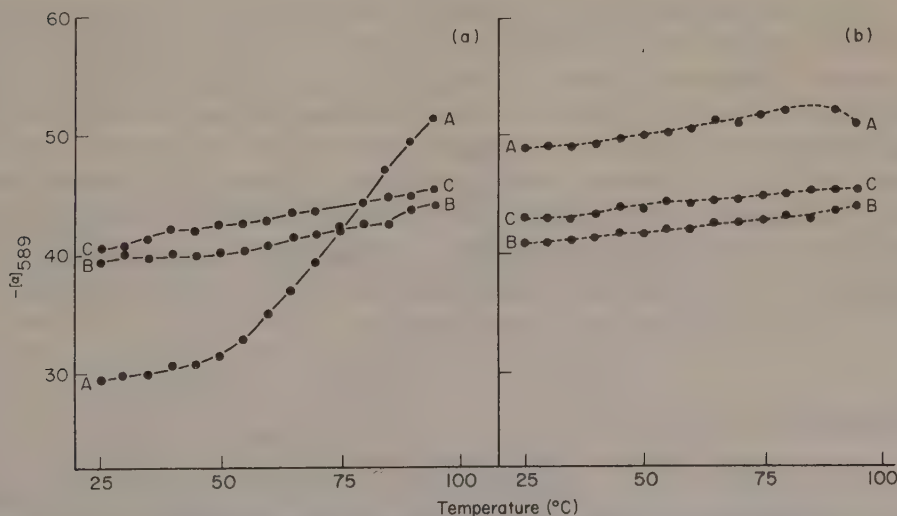


Figure 4. Specific optical rotation of ovalbumin in 40 mM sodium dodecylsulphate (SDS) as a function of temperature at different pH values. (a) Heating; (b) cooling. A, pH 5; B, pH 7; C, pH 9.

resembles the one of the protein solution without amphiphile (cf. Fig. 1a) both on heating (Fig. 4a) and cooling (Fig. 4b). The transition is, however, less sharp and the midpoint is at a somewhat higher temperature. Consequently, the effect of SDS on the conformation of ovalbumin at acidic pH values is moderate in comparison with neutral and alkaline pH values.

In order to avoid thermal aggregation at pH 3 the concentration of SDS was increased to 47 mM. This concentration corresponds to the same ratio of amphiphile : protein (w/w) as 40 mM CPC. The optical rotation *versus* temperature was almost identical to the one obtained at pH 5 and 40 mM SDS (Fig. 4a). Thus, with an addition of SDS no reversibility of the heat-induced unfolding is obtained at pH 3.

The observed difference between ovalbumin and the two amphiphiles used in this investigation might partly be explained by differences in amphiphile monomer concentration. Cetylpyridium chloride (CPC) has a critical micelle concentration (cmc) of 0.9 mM at 25°C, while SDS at the same conditions has a cmc of 8 mM (Mukerjee & Mysels, 1971). The lower monomeric concentration of CPC might be one important restriction for a co-operative binding of the monomeric amphiphile which seems to be the cause of the denaturation seen with SDS (cf. Makino, Reynolds & Tanford, 1973). The importance of micelle interaction, which has been reported for nonionic amphiphiles (Clarke, 1975; Bernath & Vieth, 1972), could therefore not be excluded in the case of CPC.

Effect on resolubility

A high solubility of dried powders is often important in food technology. Thus, in order to illustrate to what extent functional properties of a protein could be affected by addition of an amphiphile, the effect of CPC on the solubility of ovalbumin at pH 4.7 after a heating-cooling cycle was examined. To a solution of ovalbumin as added CPC to a molar ratio of 72. As a reference, an ovalbumin solution was used, to which no CPC was added. Both solutions were heated to 95°C, cooled and freeze dried. The dried samples were redissolved in distilled water and after 3 hr centrifuged at 8000 *g* for 30 min. The solubility was measured as the absorbance of the supernatants at 280 nm. Ovalbumin without CPC was soluble only to a marginal extent (0.6%), while ovalbumin in presence of CPC was completely soluble (100%), i.e. the addition of CPC brings about a complete resolubility of the protein after a very strong thermal treatment.

Conclusion

Addition of CPC in high concentrations, i.e. well above the cmc of the amphiphile, to ovalbumin at room temperature induces no increase in specific optical rotation. This amphiphile seems therefore not to cause a less ordered protein structure. This conclusion is supported by the circular dichroism measurements.

Conformational changes are registered when heated, however, but the thermal transition occurring without added amphiphile was to a large extent reduced in the presence of CPC. Furthermore, the transition induced by the thermal treatment was reversed on cooling. No evidences could be found from OR or CD measurements for differences in conformation between the unheated ovalbumin-CPC complex and the one which has been exposed to the heating-cooling cycle.

Work is in progress to examine the effect of different amphiphiles on various proteins in order to elucidate if other amphiphiles could be found which have similar effects as those registered for the amphiphile used as a model in this investigation, and if this effect also is applicable to proteins in general. In these studies, methods to study conformational changes complementary to those included here are used.

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IV

EFFECT OF CATIONIC AMPHIPHILES AND TEMPERATURE ON
LYSOZYME CONFORMATION

by

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EFFECT OF CATIONIC AMPHIPHILES AND TEMPERATURE ON LYSOZYME CONFORMATION

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ABSTRACT

The structural effect of the three low molecular weight cationic amphiphiles, dodecylammonium chloride (DAC), hexadecylpyridinium chloride (HPC) and hexadecyllysine ester dihydrochloride (HLDC) on lysozyme at different temperatures was investigated by means of calorimetical and optical methods. These amphiphiles are characterized by high (DAC) and low (HPC) critical micelle concentration in electrolyte-free solution, respectively, while HLDC forms liposomes in water. None of the amphiphiles at any concentration investigated (0.05-0.6 g amphiphile/g protein) significantly changed the native characteristics of the protein at 25°C. The results from the structural methods employed were confirmed by enzymatic activity measurements. At increased temperatures the effect on lysozyme was dependent on the association state of the amphiphile. Complete inhibition of thermal aggregation was the only effect registered at amphiphile concentration below cmc, while concentrations near and above the critical micelle concentration, also influenced the thermally induced unfolding of lysozyme. The amplitude of the major thermal transition was significantly lowered, while only minor structural changes not affecting the enzymatic activity were observed below this temperature. The postdenaturation structure of lysozyme in presence of the cationic amphiphiles used thus seems to exhibit a higher degree of order compared to lysozyme without amphiphile.

INTRODUCTION

Globular, water-soluble proteins and polar lipids co-exist in many biological and technological systems. Their mutual interaction might markedly influence protein conformation and thereby its functionality and biological activity. As thermal treatments are a frequent process parameter, the influence of polar lipids on thermally induced processes of a protein is of interest. Thermal inactivation due to aggregation and irreversible unfolding is the most important restriction for the use of biologically active proteins in industrial applications (1). These processes might be influenced by addition of appropriate polar lipids.

Synthetic low molecular weight amphiphiles have been used as model system for naturally occurring polar lipids. The influence of long-chain alkyl sulphates (with 12 or more carbon atoms) on globular proteins is

well characterized (2,3). Sodium dodecyl sulphate (SDS) is known to bind to most globular proteins in a stoichiometric weight-to-weight manner (4), thereby creating a refolded (5,6) complex with a similar geometry, regardless of the shape of the native protein (7). It is also well established that SDS interacts with proteins only in the monomeric state (4).

Interactions between nonionic amphiphiles and proteins have also been extensively studied. These amphiphiles are known to bind to membrane proteins without disruption of the native conformation or loss in biological activity (8). However, no interaction with water-soluble globular proteins, with the exception of serum albumin (9), is reported. The properties of complexes formed between water-soluble proteins and amphiphiles not inducing major conformational changes of the protein have not received as much attention in literature (10) as the SDS-protein complexes. One group of low molecular weight amphiphiles that seems to be of interest in this connection is the long-chain cationic ones (11). There are reasons to believe that cationic amphiphiles interact with proteins in a dissimilar way to the anionic ones. In a study on solubilization of water-insoluble organic compounds, Birdi and Steinhardt (12) have shown that complexes formed between cationic amphiphiles and proteins exhibit different hydrophobicity than complexes formed between SDS and proteins.

The present study has been focused on the effect of three cationic amphiphiles - hexadecylpyridinium chloride (HPC), dodecylammonium chloride (DAC) and hexadecyllysine ester dihydrochloride (HLDC) - on lysozyme at different temperatures. These amphiphiles exhibit different association behaviour in aqueous systems: HPC and DAC form micelles at low concentrations, with reported critical micelle concentrations of 0.9 mM (0.31 g/l) and 14 mM (3.1 g/l), respectively (25°C, distilled water) (13). HLDC differs in aggregation behaviour by forming liposomes in water at low concentrations, i.e. the monomeric solubility of HLDC is very low. Furthermore, HLDC has a chain melting temperature of 60°C, which is higher than for HPC and DAC (Krafft points of 12-15°C in distilled water).

The structural methods employed have been circular dichroism (CD), differential scanning calorimetry (DSC), specific optical rotation (OR), and enzymatic activity measurements.

EXPERIMENTAL

Materials

Hen egg white lysozyme (3 x crystallized lot nr 57 C-8025), Micrococcus lysodeikticus (lot nr 107C-0062) and hexadecylpyridinium chloride

(lot nr. C-8002) were purchased from Sigma Chemical Company. Dodecylammonium chloride (lot nr A3D) was obtained from Eastman Company. Hexadecyllysine ester dihydrochloride was kindly supplied by Dr. Kåre Larsson.

Lysozyme was dialyzed against distilled water for 24 h and subsequently freeze-dried to constant weight. No other egg white proteins could be detected in the preparation by SDS gel electrophoresis.

Methods

Circular Dichroism

CD-measurements were performed on a JASCO J40 dichrograph and a Jobin Yvon dichrograph Mark IV in the wavelength range of 340-190 nm. Scanning rates were 5 nm/min for JASCO J40 and 30 nm/min with 6 cycles for each sample for the Jobin Yvon instrument. Baselines were determined using either water or the corresponding amphiphile solution. Results are expressed as mean residue ellipticities ($\text{degree} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$), using the value 111.6 for the mean residue weight of lysozyme. Cell width of 0.01 and 0.1 cm and a protein concentration of 2.33 g/l were used.

Presented spectra are the mean of at least four independent measurements. The reproducibility of the recordings was $\pm 2\%$ both in the near and in the far UV-region.

Specific Optical Rotation

OR-measurements were carried out on a Perkin Elmer 241 polarimeter equipped with a DA converter, allowing continuous registration of the signal. Measurements were made at the sodium D-line (589 nm) in a thermostated polarimeter cell (1.0 dm, 5 ml). The cell was connected to a circulating water bath equipped with an electronic heat gradient control (Heto, Denmark, type 02 PH 623). A heating rate of 1 K/min and a protein concentration of 9.3 g/l was used. The presented OR-curves are the mean of three independent measurements. Data are expressed as specific optical rotation with an accuracy of $\pm 0.5^\circ$.

Differential Scanning Calorimetry

DSC measurements were carried out on a Perkin Elmer DSC-2C, at a scanning rate of $10^\circ\text{C}/\text{min}$. Coated aluminium sample pans (Du Pont no 900.796.901 and 900.790.901) with a sample volume of 20 μl were used. As reference, a sealed sample pan with an equal amount of water, was used. A protein concentration of 9.3 g/l was employed. A characteristic temperature, T_m , is defined as shown in Fig. 3. The reproducibility of T_m and the

apparent heat of denaturation, ΔH_{app} were $\pm 0.5^{\circ}\text{C}$ and 0.2 cal/g, respectively. Presented results are the mean of at least three independent measurements.

Enzymatic Activity

Enzymatic activity of lysozyme was measured in phosphate buffer (ionic strength 0.01) with a suspension of lyophilized *Micrococcus lyso-deikticus* as substrate, according to Chang and Carr (14). Stock solutions contained 2.33 g lysozyme/l, while the concentration during assay was 0.04 g/l. The results are the mean of at least three measurements with a reproducibility of $\pm 5\%$.

Preparation of solutions

All protein solutions were made up in double distilled deionized water as described earlier (11). The amphiphile solutions were all in melted state before addition to the protein solution. All experiments in this investigation were carried out at pH 5, where lysozyme carries a positive net charge of about 10. Higher pH-values induce thermal aggregation at lower temperatures, and also facilitates the dimerization process of lysozyme (15).

RESULTS

Circular Dichroism

The CD-spectra of lysozyme in the far and near UV-regions are given in Fig. 1 (curve 1). The far UV-spectrum, which provides information about the secondary structure, shows a negative plateau in the range of 220 nm, a negative peak at 208 nm and a positive one at 193 nm (not shown in the figure). Analysis according to Chang et al. (16), using the known conformation of 30 proteins as reference, reveals 45% α -helix, 5% β -sheet, 22% β -turn and 28% other structure. By X-ray the secondary structure has been reported to 45% α -helix, 19% β -sheet and 14% β -turn (17). The figure on the α -helical content coincides, while the β -structure content estimated from CD-measurements shows a rather large deviation from the X-ray value.

The near UV-spectrum, which gives information about the tertiary structure, is shown in Fig. 1b and is in agreement with the spectrum reported by Goux and Hooker (18), who have also given a thorough analysis of the spectrum.

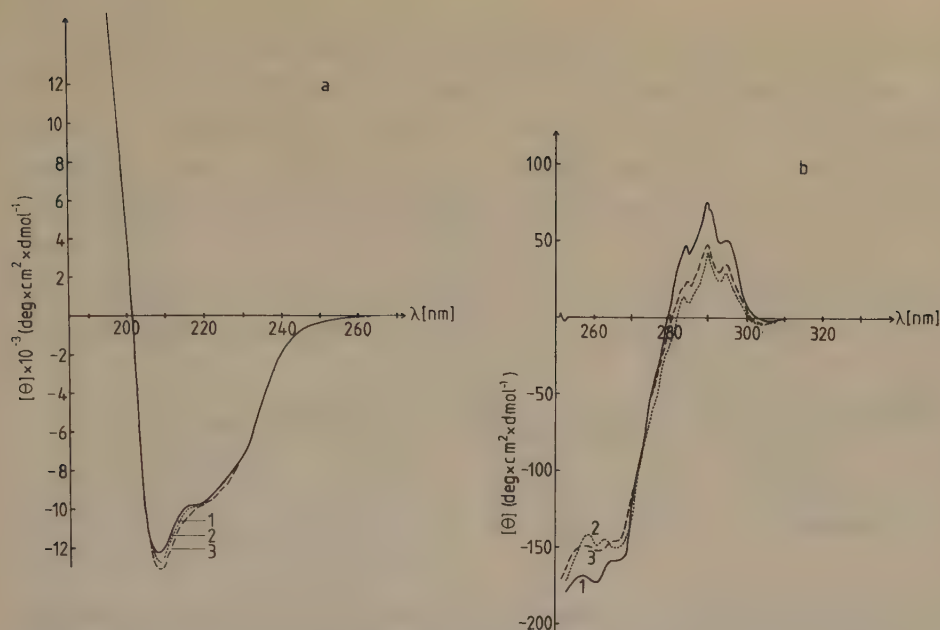


FIG. 1. Circular dichroism of lysozyme at pH 5 with various cationic amphiphiles in a) the far UV-region, b) the near UV-region. Curve 1: Pure lysozyme, lysozyme in 0.93 g HLDC/l, lysozyme in 0.93 g DAC/l, lysozyme in 0.23 g HPC/l. Curve 2: Lysozyme in 0.93 g HPC/l; Curve 3: Lysozyme in 3.48 g DAC/l. Protein concentration was 2.33 g/l.

HLDC, at a concentration of 0.93 g/l (0.40 g per g lysozyme), HPC and DAC at concentrations below cmc (0.23 and 0.93 g/l, respectively, corresponding to amphiphile-to-protein weight ratios of 0.10 and 0.40 g per g) have no influence on the CD-spectrum of lysozyme, neither in the far nor in the near UV-region. All these conditions are given by curve 1 in Fig. 1. HPC and DAC well above cmc (curve 2 and 3) give rise to somewhat altered spectra, especially in the near UV-region, where both amphiphiles reduce the intensity of the CD-signal. The position and relative intensities of the bands are, however, only slightly effected by the amphiphiles. In the far UV-region there is a small increase (2-5%) of the CD-signal in the wavelength range of 230 to 204 nm. The positive peak at 193 nm is unaffected by the presence of the amphiphiles. An increase of the HPC concentration to 3.48 g/l does not further change the spectrum.

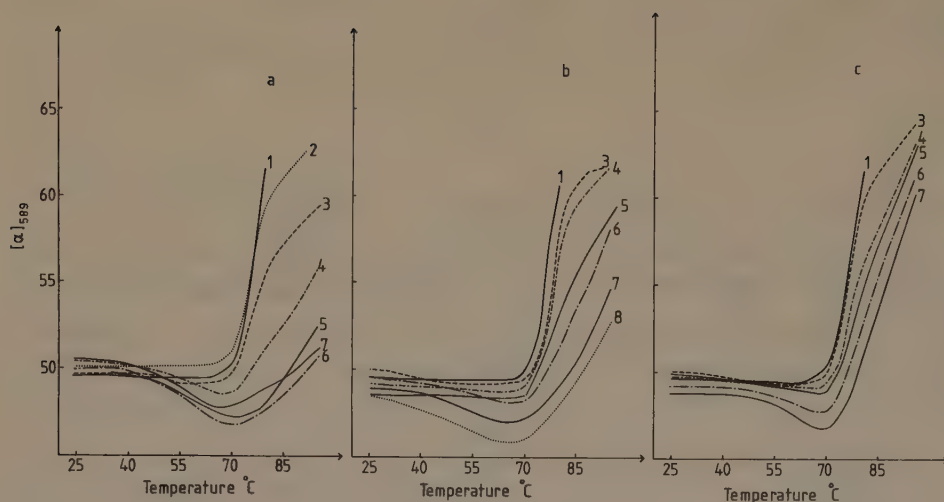


FIG. 2. Specific optical rotation of lysozyme at pH 5.0 with added HPC (a), DAC (b) and HLDC (c). Amphiphile concentrations are 0 (curve 1), 0.465 g/l (2), 0.93 g/l (3), 1.86 g/l (4), 2.79 g/l (5), 3.72 g/l (6), 4.69 g/l (7) and 5.58 g/l (8). Protein concentration was kept constant at 9.3 g/l.

Specific Optical Rotation

Optical rotation of lysozyme versus temperature is shown in Fig. 2 curve 1. The thermally induced unfolding process, which is seen as an increase in levorotation, commences at 65°C. During the unfolding process, lysozyme aggregates, which makes measurements by optical methods at temperatures higher than 80°C impossible. The aggregation process is, however, inhibited by the added amphiphiles, even at low concentrations.

Optical rotation of lysozyme was investigated at amphiphile concentrations in the range of 0.465 to 5.58 g/l (i.e. weight ratios of 0.05 to 0.6 g/g protein). Analogous with the CD-results, the most pronounced effect on lysozyme seen by the OR-curves originates from HPC and DAC added at concentrations near and above cmc. It can be seen from Figs. 2a and b that, without influencing the OR-value at 25°C, there is a gradual decrease in the levorotation at predenaturation temperatures. This decrease is more pronounced the higher the amphiphile-to-protein weight ratio is. The OR-value at 95°C is also drastically reduced. At amphiphile concentration below the cmc, the influence on the OR-curve is much less; the OR-value at post-denaturation temperatures are somewhat lowered but the decrease in OR-value at predenaturation temperatures is not seen.

HLDC has a somewhat different effect on the temperature dependence of the optical activity of lysozyme (Fig. 2c). The gradual decrease in OR-values at predenaturation temperatures is registered at higher amphiphile-to-protein weight ratios (from 0.40 g/g), and the decrease in post-denaturation OR-value is not as pronounced as with the micelle forming amphiphiles.

The OR-curves for all combinations of lysozyme-amphiphiles are reversible on cooling.

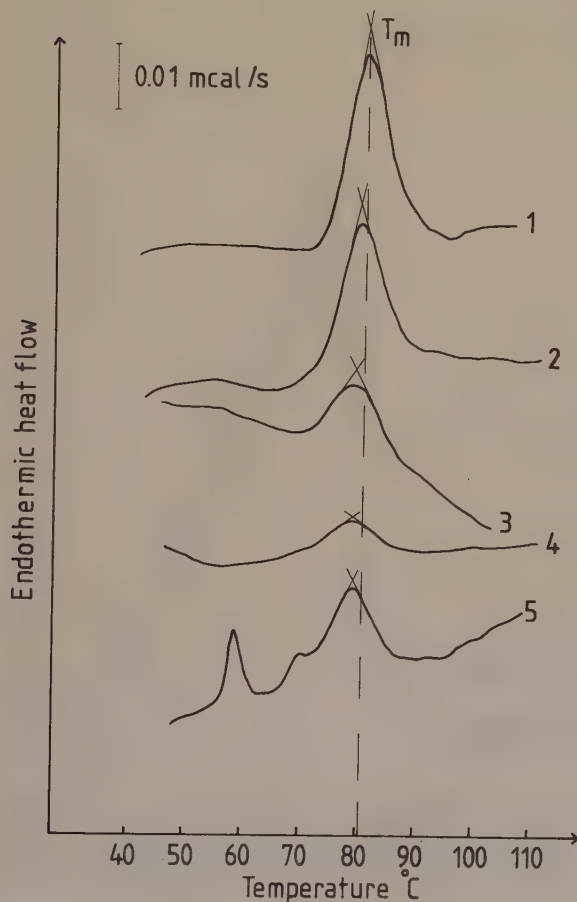


FIG. 3. Typical DSC-thermograms of lysozyme and cationic amphiphiles. Lysozyme (curve 1), lysozyme in 1.86 g DAC/l (2), lysozyme in 2.79 g DAC/l (3), lysozyme in 1.86 g HPC/l (4), and lysozyme in 3.72 g HLDC/l (5). Protein concentration was 9.3 g/l.

Differential Scanning Calorimetry

A DSC thermogram of lysozyme is shown in Fig. 3, curve 1. Thermal unfolding of pure lysozyme is characterized by an apparent enthalpy of 7.7

cal g^{-1} and a T_m of 80.5°C when heated at $10^\circ\text{C min}^{-1}$. Addition of amphiphile lowers the apparent denaturation enthalpy, while the denaturation temperature is only slightly influenced. Some typical DSC-tracings are shown in Fig. 3. The thermogram of lysozyme with added HLDC shows some interesting features. The denaturation peak is accompanied by an additional transition peak, probably originating from associated HLDC, while free HLDC shows a transition around 60°C .

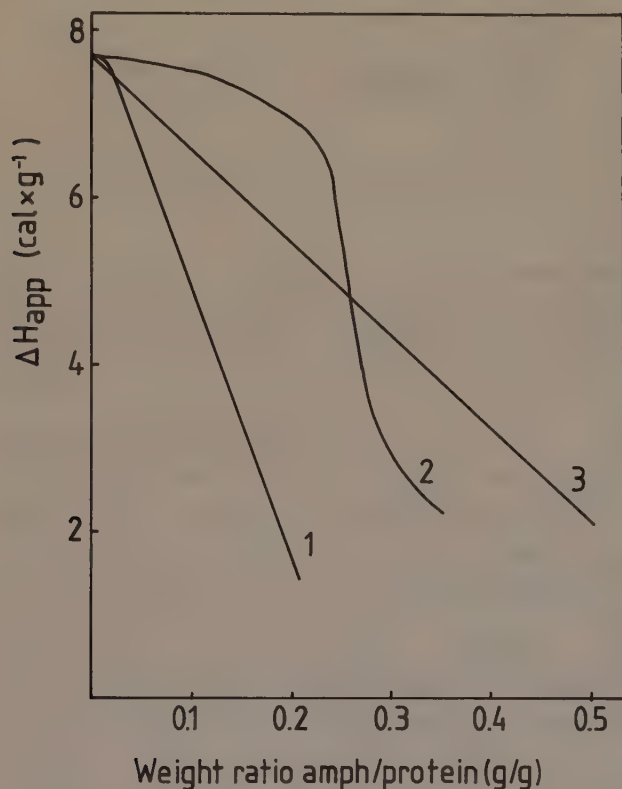


FIG. 4. Apparent heat of denaturation, ΔH_{app} , of lysozyme at pH 5.0 versus concentration of amphiphile. Curve 1 shows lysozyme with added HPC, curve 2 lysozyme with added DAC and curve 3 lysozyme with added HLDC. Protein concentration was kept constant at 9.3 g/l .

The apparent denaturation enthalpies of lysozyme are summarized in Fig. 4. The effect on the thermal unfolding process achieved by the micelle forming amphiphiles arises at amphiphile concentration close to cmc. This is most clearly seen from the measurements with DAC, which exhibits the highest cmc. HLDC, however, lowers the denaturation enthalpy linearly with increasing concentration. The denaturation temperature is,

however, only lowered by 2°C by the highest HLDC concentration investigated.

Enzymatic Activity

The effect of the studied amphiphiles on the enzymatic activity of lysozyme is shown at different temperatures in Fig. 5. Amphiphile concentrations are the same as in the circular dichroism experiments. None of the amphiphiles, at any concentration investigated, decrease the activity of lysozyme. It can be seen that the activity is somewhat increased, especially at higher temperatures, but without any change in temperature optimum.

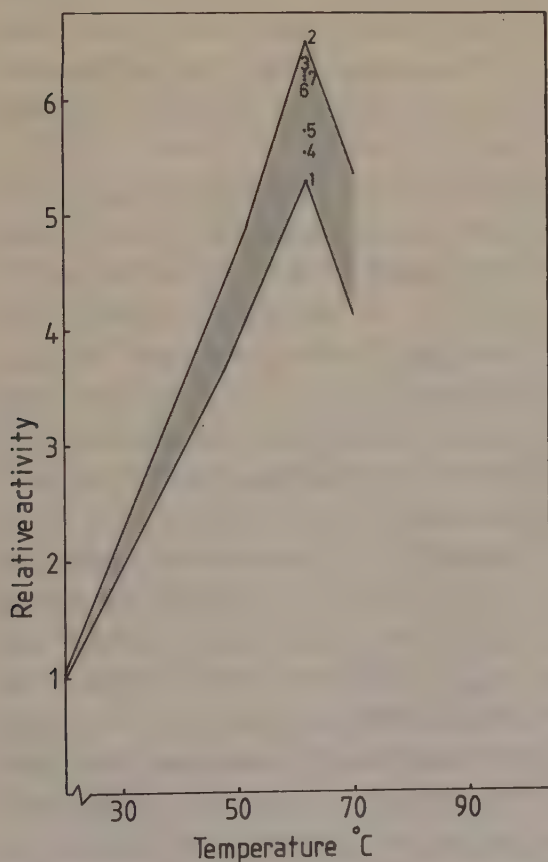


FIG. 5. Relative enzymatic activity of lysozyme versus temperature. Lower curve (1): lysozyme without amphiphile, upper curve (2): lysozyme in 0.23 g HPC/l. The curves 3-7 are situated within the shadowed area, with the same slopes as curves 1 and 2. The points indicate the relative activity at the temperature optimum - 3 and 4: lysozyme in 0.93 and 3.48 g HPC/l, respectively; 5 and 6: lysozyme in 0.93 and 3.48 g DAC/l, respectively; 7: lysozyme in 0.93 g HLDC/l.

DISCUSSION

The refolding effect of anionic amphiphiles, especially SDS, on protein conformation is not seen by the cationic amphiphiles at the conditions used in this investigation. At room temperature no detectable effect on lysozyme conformation, as measured by circular dichroism both in the far and near UV-region, was observed by addition of HLDC or of HPC and DAC below cmc. HPC and DAC added to concentrations near and above the cmc, however, cause a decrease in the intensity of the CD-signal in the near UV-region, indicating a minor influence on the tertiary structure of lysozyme. The native qualities, as measured by enzymatic activity of lysozyme, however, is not deteriorated by addition of the cationic amphiphiles at any concentration. Instead, an increase in activity is seen by addition of the amphiphiles which might indicate a decrease in the dimerization process of lysozyme (15,20). Furthermore, no time dependence of the protein amphiphile interaction, as reported by Hayashi et al. (19) was found within 24 hours.

The thermally induced aggregation and unfolding processes are affected by the amphiphiles. With the micelle-forming amphiphiles, these effects are highly dependent on the concentration relative to cmc. At concentrations below the cmc, inhibition of aggregation is the main effect. Amphiphile concentrations near and above the cmc also influence the thermally induced conformational transitions of lysozyme, manifested at predenaturation, denaturation and postdenaturation temperatures. The decrease in levorotation at predenaturation temperatures by higher amphiphile concentrations is difficult to elucidate, but is assumed to be due to a higher degree of hydrophobic interaction with increasing temperature. The major heat-induced transition, measured by both calorimetical and optical methods, is substantially lowered. This leads one to conclude that the structure at postdenaturation temperatures is quite different from that obtained without additive. The thermal denaturation of pure lysozyme gives rise to an unfolded state, indistinguishable from that obtained in 6 M guanidine hydrochloride (21). This unfolded state is considered to lack ordered structure. As no substantial unfolding of lysozyme was detected in the presence of DAC and HPC at predenaturation temperatures, the amplitude of the heat-induced transition indicates a less unfolded protein structure at postdenaturation temperatures in presence of these amphiphiles.

Lysozyme exhibits a high degree of reversibility on heat denaturation. At conditions used in this investigation, the reversibility for pure lysozyme was about 80%. In the presence of cationic amphiphiles, the reversibility increases to around 95%, probably due to the inhibition of intra- and interchain interactions. This effect was also seen earlier for a completely irreversible protein, ovalbumin (11).

The presented results indicate that the interaction between lysozyme and the micelle-forming cationic amphiphiles might occur in two stages, depending on the cmc. In the concentration range below cmc the amphiphile, in monomeric or dimeric form (22), is assumed to bind to oppositely charged groups of the protein. This will increase the electrostatic repulsive force, thereby increasing protein solubility at the acidic side of the isoelectric point. The second stage interaction, occurring when the total amphiphile concentration approaches the cmc, might be seen as analogous to micelle formation. The structure of lysozyme is characterized as highly charged, but also as hydrophobic. Shrake and Rupley (23) have concluded that more than 50% of the solvent-exposed surface of lysozyme is, in fact, hydrophobic. The hydrophobic surfaces of lysozyme, together with a possible increased relative hydrophobicity resulting from the first stage binding, might act as hydrophobic interfaces in the solution, facilitating a gross interaction between the protein and the low-molecular weight amphiphile, as cmc is approached. Since this process starts slightly below the cmc, a gain in entropy in forming "mixed micelles" between the protein and the amphiphile, compared to ordinary micelle formation can be assumed. This hypothesis is supported by the fact that the effect seen on protein denaturation is independent of protein concentration (in the range 0.1-5.0%) but is dependent on the cmc of the amphiphiles (0.31 g/l for HPC and 3.1 g/l for DAC). Only the exterior parts of lysozyme seem to be involved in the interaction, since there is no reorganization of the secondary structure. This interaction differs from SDS-protein interactions, which involve a consecutive unfolding and formation of new binding sites from the interior of the protein (24,25).

The stability of lysozyme against unfolding by the cationic amphiphiles is assumed to derive from the balance of the hydrophobic interaction by the electrostatic repulsive force. As long as this balance is maintained, the interaction is limited. The measurements reported here were carried out in an electrolyte free medium. When increasing the ionic strength, i.e., decreasing the electrostatic repulsion and thereby strengthening the hydrophobic association (26), the complex formed between

lysozyme and HPC shows a decreased thermostability. Earlier studies on the association of cationic amphiphiles to lysozyme, carried out at higher ionic strength also report a destabilization of the protein structure (27).

HLDC, having a very low monomeric solubility, exhibits a weaker influence on lysozyme compared to the micelle-forming amphiphiles. It can be assumed that the lysine moiety of HLDC increases the repulsive force, thereby decreasing the hydrophobic interaction. However, some interaction occurs, as evidenced by the inhibition of aggregation and the suppression of the heat denaturation of lysozyme.

The findings reported here point to the diversity of the effect of synthetic polar lipids on protein structure and function. The non-aggressive effect of cationic amphiphiles on protein conformation has earlier been described for ovalbumin (11). Myoglobin has, on the other hand, been reported to be unfolded by HPC (28). Obviously, no general conclusion on protein-amphiphile lipid interactions can be drawn from these results. When a specific effect is desired, an appropriate balance between the attractive and repulsive forces must be found and adapted to each protein-amphiphile system. Low molecular weight amphiphiles can thus act either destabilizing on protein conformation, or increase both solubility and stability of proteins.

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V

SURFACE BEHAVIOUR OF ADSORBED FILMS FROM PROTEIN-
AMPHIPHILE MIXTURES

by

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Surface behaviour of adsorbed films from protein-amphiphile mixtures

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Abstract: The effect of interaction between proteins and amphiphiles on the surface tension reduction have been measured by the drop-volume method. The equilibrium surface tension reduction isotherms at the air/water interface of serum albumin and of ovalbumin in sodium dodecylsulphate and of ovalbumin in 1-monocaproin are reported. The surface tension reduction isotherms of the proteins in the anionic amphiphile solutions exhibit plateau regions, which have been interpreted in terms of different states of protein-amphiphile interaction in the bulk solution. Any interaction between ovalbumin and the monoglyceride is not reflected in the surface tension isotherm. At increased amphiphile concentration the protein seems to be replaced by 1-monocaproin in the surface.

Key words: Surface tension, protein-amphiphile interaction

Introduction

Interaction between proteins and other amphiphilic substances has been shown to influence technical properties of proteins in food systems. Since proteins and low molecular weight amphiphiles often coexist in systems where formation and properties of interfacial films are of fundamental importance (e.g. dairy products, dough, meat emulsions), influence of the mode of interaction between proteins and amphiphiles in the bulk solution on the surface behaviour is of interest.

The interfacial tension of adsorbed protein films in connection with functional properties has been reported [1, 2, 3]. Also the interfacial mixed films of proteins and lipids have been investigated, either by spreading films of both components [4, 5], or by examination of the penetration of one component into a surface film of the other component [6, 7].

In the present study, the equilibrium surface tension reduction of adsorbed films from mixtures of proteins and lipids possessing different interactions in bulk solution are reported. The systems investigated are bovine serum albumin (BSA) and ovalbumin (OA) in sodium dodecylsulphate (SDS) and OA in 1-monocaproin. The interaction between SDS and proteins are well documented in literature and reviewed in reference [8]. SDS is known to bind to most proteins in a nonspecific, co-operative manner, thereby gen-

erally inducing gross unfolding of the protein structure. Few proteins seem to exhibit specific binding sites for a lipid type of amphiphiles. Studies on the interaction between BSA and SDS have revealed 8–10 high affinity sites, which are both hydrophobic and electrostatic in nature [9]. The specific binding of anionic amphiphiles to BSA stabilizes the protein structure, which can be seen as an increased resistance towards thermal denaturation [10]. At SDS-to-BSA ratios above the specific binding region the interaction is nonspecific, resulting in unfolding of the protein structure. OA, on the other hand, interacts with SDS only in a nonspecific mode [11].

In the third system studied, OA in 1-monocaproin solution, none or moderate interaction is supposed to occur, since it has been shown [12, 13] that non-ionic amphiphiles only possess weak affinity, if any, to water-soluble proteins. 1-Monocaproin has interesting phase behaviour in aqueous systems. Only one liquid phase exists [14]; in water-rich systems ordinary micelles are formed and in amphiphile-rich systems the isotropic liquid phase is of the L2 type. The interfacial behaviour of this component has not yet been reported.

Materials and methods

Bovine serum albumin (essentially fatty acid free, lot nr. 16C-7201) and hen egg white ovalbumin, free from the S-variant [15] (lot nr. 116C-8220) were purchased from Sigma

Chemical Co. The specifications given by the manufacturer of the proteins were checked by differential scanning calorimetry. Both proteins gave single transition peaks revealing no fatty acids in the BSA preparation [10] and no contamination of the S-variant in the ovalbumin preparation [16]. SDS-polyacrylamide electrophoresis showed single bands for both proteins. BSA was used as supplied, while ovalbumin was desalted on a column of Sephadex G-25 (medium) in ammonium bicarbonate buffer. After chromatography and dialysis against diluted buffer, the protein was freeze-dried to constant weight.

Sodium dodecyl sulphate (SDS), specially pure (declared purity 99%) was from BDH Chemicals Ltd., and used as supplied. The preparation of 1-monocaproin has been described by Larsson [17]. Other reagents used were of analytical grade.

Protein solutions, made up in either phosphate buffer or in double distilled water, was diluted to final concentration (1 mg/ml) by addition of amphiphile solution of appropriate concentration. Protein and monocaproin solutions were freshly prepared, while the diluted SDS solutions were made up from a stock solution (0.5 mM). To avoid precipitation at low molar ratios of the anionic amphiphile-protein complexes, the experiments were performed at pH 5.6, i.e. at the alkaline side of the isoelectric point of the proteins. All measurements were performed at $23 \pm 0.5^\circ\text{C}$.

The time dependent surface tension decay was measured according to the drop-volume technique as outlined by Tornberg [18, 19]. The automatization procedure according to Arnebrant and Nylander [20] was employed. In this method surface tension reduction by macromolecules during adsorption at the air-water interface is measured by formation of drops of certain volumes. Time for detachment of the droplets is recorded. Surface tension calculated [19, 20] was plotted against detachment time and the value attained after 2000 seconds was set as the equilibrium value. The surface tension of the solutions is still decreasing after this period of time, but the rate of decrease is small, less than 0.05 mNm^{-1} per 100 seconds. The maximum error in surface tension values is $\pm 1.5\%$.

The drop-volume method allows measurements of solutions without further dilution, i.e. irreversibility and/or time-dependent reversibility at dilution of the complexes due to total concentration of amphiphile is avoided. In addition, adsorption to the surface created during formation of the droplet might be seen as analogous to adsorption to surfaces created in foams and emulsions, for example.

Results and discussion

The surface tension reduction ($\Delta\gamma$) by SDS of 0.05 M phosphate buffer, pH 5.6, at 23°C is indicated in figures 1 and 2. At conditions used surface tension of SDS at concentrations above 1 mM is not possible to measure, since at this concentration the critical temperature curve is surpassed. The knee-point of this curve, the Krafft point, which has been reported to $9-10^\circ\text{C}$ [21], is known to be highly sensitive to impurities, which decrease the critical temperature, and to added salts, which is thought to increase this temperature [22].

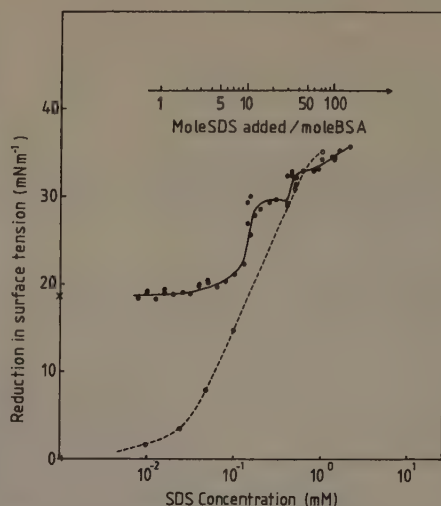


Fig. 1. The equilibrium surface tension reduction isotherm of SDS (broken line) and of $13.4 \mu\text{M}$ BSA in SDS solution (solid line). The surface tension reduction of $13.4 \mu\text{M}$ BSA without SDS is indicated on the ordinate. Measurements were performed in 0.05 M phosphate buffer, pH 5.6 at 23°C .

The equilibrium value of reduction in surface tension by $13.4 \mu\text{M}$ BSA of the phosphate buffer is 19.4 mNm^{-1} . The protein concentration is well above

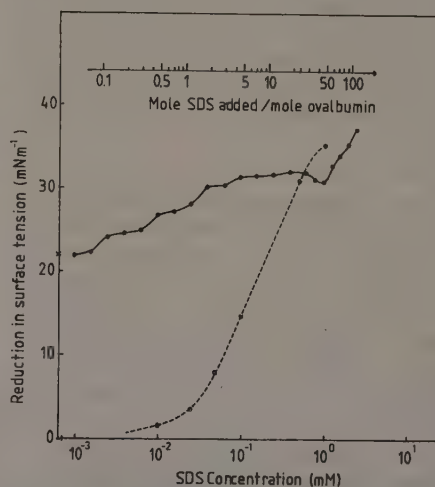


Fig. 2. The equilibrium surface tension reduction isotherm of SDS (broken line) and of $21 \mu\text{M}$ ovalbumin in SDS solution (solid line). The surface tension reduction of $21 \mu\text{M}$ ovalbumin without SDS is indicated on the ordinate. Experimental conditions as in figure 1.

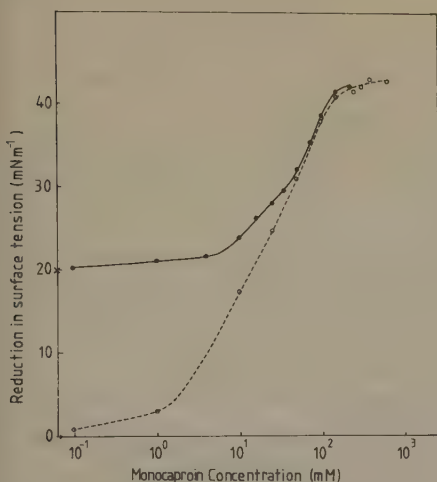


Fig. 3. The equilibrium surface tension reduction isotherm of 1-monocaproin (broken line) and of 21 μM ovalbumin in 1-monocaproin solution (solid line). The surface tension reduction of ovalbumin without the amphiphile is indicated on the ordinate. Measurements were performed at 23°C in double distilled water, pH adjusted to 5.6 using 1.0 N NaOH

the surface saturation concentration. $\Delta\gamma$ of 13.4 μM BSA as a function of total SDS concentration is shown in figure 1. At SDS-to-BSA ratios approximately corresponding to the specific binding region, no significant effect on surface tension can be seen. The specific binding of anionic amphiphiles to serum albumin is supposed to occur to hydrophobic areas situated close to positively charged amino acid residues [9]. This binding does not seem to influence the saturation surface tension of the protein. Further, the association constant is high and the concentration of unbound SDS in the solution is low (below 10^{-5}M [8]). At SDS concentrations slightly above the specific binding region, an abrupt increase in $\Delta\gamma$ is observed. This might be caused by the increased concentration of unbound SDS. Furthermore, at SDS concentrations below those required for co-operative binding (approximately 0.1 mM [13]) an electrostatic binding of the anionic amphiphile to positively charged amino acid residues of the protein might occur, resulting in formation of more surface active complexes. Further increase of SDS concentration gives rise to plateau regions, indicating constant concentration of unassociated amphiphile. This behaviour is to be expected at the co-operative association of amphiphile to protein, occurring over a very narrow range of free SDS concentration. This binding has also been shown to be

accompanied by an unfolding of the protein structure [8, 10]. But still both unbound SDS and the complex formed are supposed to contribute to the surface tension reduction, since the $\Delta\gamma$ -value at the plateau is higher than the $\Delta\gamma$ -value for pure SDS at equal concentrations.

At SDS concentrations above approximately 1 mM the surface tension reduction seems to be mostly governed by the amphiphile. Either no further interaction to the protein occurs, or the free SDS concentration is sufficiently high to create surface films that cannot be penetrated by the negatively charged protein-amphiphile complex.

The reduction in surface tension by 21 μM ovalbumin of the phosphate buffer is 22 mNm^{-1} , i.e. a slightly higher value than obtained by BSA. The $\Delta\gamma$ isotherm of 21 μM ovalbumin as a function of SDS concentration is shown in figure 2. Except for the region of specific binding in the BSA-SDS surface tension isotherm, the general pattern is similar. At low SDS concentration, where no co-operative binding of SDS to OA is supposed to occur, there is a gradual increase in $\Delta\gamma$. This phenomenon can be caused by a synergistic effect, arising from the possibility of a more optimal packing of SDS and protein segments at the surface compared to the individual components. Further, an electrostatic interaction between the protein and SDS would increase the hydrophobicity of the protein. Most probably, the increase originates from a combination of both these processes. At increased SDS concentration, a plateau value of $\Delta\gamma$ is reached, where, in analogy to the BSA-SDS system, a co-operative binding of SDS to OA, associated with unfolding of the protein, is supposed to occur. For OA this region starts at a lower total SDS concentration, while the effect of interaction on the surface tension isotherm is completed at around the same total concentration of amphiphile.

Figure 3 shows the surface tension reduction isotherm of 1-monocaproin in double distilled water. The association concentration of this compound is high, the critical micelle concentration is estimated to 160 mM. The saturation value of $\Delta\gamma$, 42 mNm^{-1} , though, is in the same order of magnitude as obtained by long-chained monoglycerides. The presence of ovalbumin at a concentration of 21 μM only displaces the surface tension isotherm to the $\Delta\gamma$ -value of the pure protein in water (20 mNm^{-1}). At amphiphile concentrations below 5 mM only a slight increase in $\Delta\gamma$ is seen, i.e. the free nonionic amphiphile does not seem to contribute to the surface tension reduction. This might be interpreted as an interaction between the protein and monocaproin not influencing the sur-

face tension decrease. But since no interaction between a nonionic amphiphile and water-soluble proteins is supposed to occur [12, 13], it is more likely that the protein, being the most surface-active component at low concentration of this amphiphile, dominates the surface film. Further, no synergism in surface tension is reported for mixtures of nonionic amphiphiles [23]. The surface tension reduction at higher monocaproin concentrations seems to be mostly governed by the amphiphile, the protein merely being replaced in the surface film by the monocaproin. At surface tension reduction above 30 mNm^{-1} the isotherm in presence of protein is identical to the one seen by pure monocaproin, indicating that all the protein molecules have been completely squeezed out from the surface film. This can also be seen as a support for the proposed lack of interaction between the protein and amphiphile since there is no displacement of the isotherm towards higher amphiphile concentration in presence of protein. Such a displacement is clearly seen in the other two isotherms presented.

Conclusions

From the surface tension isotherms reported it can be generalized that interactions between protein and amphiphile in the bulk solution are closely related to adsorption behaviour at the air/water interface. Association of lipid-like substances to proteins results in plateau regions of the isotherms, within which neither changes in the amphiphile concentration, nor the supposed consecutive unfolding of the protein structure is reflected in the $\Delta\gamma$ -value. Replacement of protein in the surface film by a lipid type of amphiphile, when no interaction occurs, was seen in the monocaproin-ovalbumin isotherm, and also in the SDS-protein isotherms at sufficiently high amphiphile concentrations. Similar effects have also been observed in the membrane of the fat globule in milk [24], by addition of a nonionic amphiphile at emulsification.

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VI

EFFECTS OF AMPHIPHILES ON TRYPSIN ACTIVITY AND CONFORMATION

by

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EFFECTS OF AMPHIPHILES ON TRYPSIN ACTIVITY AND CONFORMATION

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ABSTRACT

Amphiphiles with different head groups (sulphate, carboxylate, pyridinium, ammonium, trimethylammonium and oxyethylene) and chain lengths (octyl, dodecyl and hexadecyl) were used to study the effect of amphiphiles on trypsin esterolytic activity, stability and conformation. The electrophoretic mobility of autohydrolysed trypsin samples was used complementarily with activity measurements to follow the deactivation of trypsin. It is concluded that amphiphile-trypsin interaction is highly dependent on the hydrophilic moiety but also to some extent on the hydrocarbon chain length. The anionic amphiphiles exhibit a more aggressive influence on trypsin conformation than the cationic ones. With the exception of potassium dodecanoate, the examined anionic surfactants decrease trypsin activity, while both activity and stability of trypsin are increased by the cationic ones. These effects are correlated to conformational changes of the protein, as measured by circular dichroism.

INTRODUCTION

Protein conformation and function are sensitive to the presence of various co-solutes, as e.g. low molecular weight amphiphiles. The binding equilibria of anionic surfactants to globular proteins, with concomitant reorganization of the native conformation, have been extensively studied (1,2). The same applies for the solubilization of native membrane proteins by nonionic surfactants (3-7).

Amphiphiles are commonly used additives in detergents, cosmetics, pharmaceutical products and in foods. In food different types of low molecular weight amphiphiles also occur naturally. In many of those systems, amphiphilic compounds coexist with proteins.

Amphiphiles exhibit a broad scale of interaction with globular proteins, depending both on the nature of the components and on external conditions such as temperature, ionic strength, pH, etc. The knowledge of the effect of various surfactants on native qualities, e.g. enzymatic

activity, as well as on technological properties of globular proteins, is still incomplete. It was, however, recently shown that both physico-chemical and technological properties of globular proteins can be affected by cationic surfactants (8). Since these effects were obtained without any major unfolding of the protein at room temperature, it was of interest to extend the investigation to include the effect of various surfactants on an enzyme.

The present work is thus a survey of the effect of various amphiphiles on trypsin activity. The esterolytic activity of trypsin is examined as a function of incubation time. Circular dichroism is used to relate the effects obtained on trypsin activity to the influence of the surfactants on trypsin conformation.

MATERIALS AND METHODS

Trypsin (bovine pancrease, type III, 2 x crystallized, lot nr. - 80F-8065), sodium dodecylsulphate (SDS) and hexadecylpyridinium chloride (HPC) were obtained from Sigma Chemical Co., and hexadecyl-trimethylammonium bromide (HTAB) and sodium hexadecylsulphate (SHS) from Merck. Dodecylammonium chloride (DAC) and Triton X-100 were from Eastman Kodak Company. These compounds were used as supplied. The potassium salts of dodecanoic (KC_{12}) and hexadecanoic acids (KC_{16}) (BDH Chemical Ltd.) were prepared by neutralization in nonaqueous medium according to Mandell and Ekwall (9). Hexadecylammonium chloride (HAC) was prepared by allowing gaseous chloride to pass through a solution of hexadecylamine (purum, Fluka) dissolved in ether. Dodecylpyridinium chloride (for synthesis, Merck) was recrystallized from acetone at low temperature ($-20^{\circ}C$).

Trypsin solutions were prepared from a daily stock solution (4.0 g/l dissolved in 0.001 M HCl), which was diluted with double distilled de-ionized water to give a final concentration of 0.090 g/l. The different surfactants, dissolved in water, were added in an amount corresponding to the weight ratio of 0.40 g per g protein in all experiments. Final concentration is well below cmc for all surfactants studied. In order to prevent titration of the soaps, the pH of the enzyme solution was, prior to addition, adjusted to 8 (1.0 M NaOH). Finally, pH was adjusted to 9.0 and the enzyme solutions were kept ice-cooled until assay. Kept at this temperature, trypsin is stable for at least 10 h.

The esterolytic activity of trypsin on the synthetic substrate N-benzoyl-L-arginine ethyl ester (BAEE) (Sigma Chemical Co.) was followed by

the increase in optical density at 253 nm. The assay medium was 3.7 ml 0.05 M Tris/HCl buffer, pH 9.0, to which 100 μ l substrate solution (8.0 g BAE per l) was added. The substrate solution was kept ice-cold prior to use. The reaction was started by adding 200 μ l trypsin solution, which included the amphiphile examined. Activity values reported are the mean of three independent measurements, with a reproducibility of $\pm$ 5%.

Circular dichroism (CD) measurements were performed using a JASCO J40 dichrograph. Spectra were recorded at a scanning rate of 5 nm per minute. Baselines were determined using either water or the corresponding surfactant solution. Results are expressed as the mean residue ellipticities (degree $\times$ cm² $\times$ dmol⁻¹) using the value 103 for the mean residue weight of trypsin. The cell width used was 0.2 cm in the far UV-region and 2.0 cm in the near UV-region. The reported spectra are the mean of three recordings, at the highest sensitivity setting available (0.5 m⁰/cm). The reproducibility of the recordings was $\pm$ 5% at wave-lengths above 205 nm. At shorter wave-lengths, where scattering occurs due to stronger absorption, the sensitivity and also the reproducibility is lowered by a factor of 2.

Urea-SDS polyacrylamide gel electrophoresis (PAGE) was performed mainly according to Swank and Munkres (10) at an acrylamide concentration of 12.5% (w/v), an acrylamide : N,N'-methylenebisacrylamide weight ratio of 10:1 in 8 M urea. The electrophoresis was carried out at the constant current of 20 mA per gel slab. The time required for the reference dye to reach the bottom of the gels was 15-20 h. As reference for the molecular weight determination a polypeptide molecular weight (PMW) calibration kit (Pharmacia Fine Chemicals), consisting of the partial cyanogen bromide cleavage products of sperm whale myoglobin, was used. The trypsin samples, incubated at 30°C for different periods of time, were freeze-dried and redissolved in the electrophoretic medium.

RESULTS

Effect of Amphiphiles on the Esterolytic Activity of Trypsin

The esterolytic activity of trypsin at 20°C in the presence of various amphiphiles is reported in Table 1. Values are given as the percentage of trypsin activity without any additive. The anionic amphiphiles SDS and SHS decrease the activity of trypsin. The same effect is achieved by the long-chain fatty acid salt, KC₁₆, while the analogous dodecyl compound increases the esterolytic activity slightly. The single nonionic surfactant examined, Triton X-100, does not show any significant effect on the activity.

TABLE 1

The effect of amphiphiles on the initial rate of trypsin esterase activity at pH 9.0. The amphiphile-to-protein weight ratio is 0.40 g/g. The activity of pure trypsin is used as reference value and is normalized to 100%.

Additive	Initial rate (%)
None	100
SDS	50
SHS	50
KC ₁₂	115
KC ₁₆	50
Triton X-100	105
DPC	125
HPC	135
DAC	145
HAC	125
HTAB	130

All the cationic surfactants used increase the activity of trypsin. The activity increase is in the range of 25 to 45%, independent of the hydrocarbon chain length and the hydrophilic group.

The Time and Temperature Dependent Deactivation of Trypsin

The effect of amphiphiles on the stability of trypsin at 30°C was measured as the decline in esterolytic activity against time and is shown in Fig. 1. The activity decrease of pure trypsin is given by curve 1. Trypsin is known to autohydrolyse at pH-values above 6 (11); at pH 9 this process is rapid, with only 55% of the initial activity remaining after 2 h. After an incubation time of 24 h, 95% of the activity is lost.

The reduction in trypsin activity by the anionic surfactants SDS, SHS and KC₁₆ seen in Table 1 is intensified at 30°C at prolonged incubation time (Fig. 1A). SDS is the most effective one, causing a complete loss of activity within 1 h. The cationic amphiphiles examined, however, all increase the stability of trypsin (Fig. 1B). This effect is also achieved by dodecanoate and Triton X-100 (Fig. 1A, curve 4,6). Over a period of 2 h, KC₁₂ seems to have the most preserving effect of all the dodecyl compounds examined, while the cationic dodecyl surfactants are more effective over a

prolonged incubation time (Fig. 1B). Triton X-100 behaves similar to KC_{12} ; the loss in activity is slowed down, but not to the same extent as seen by the cationic amphiphiles.

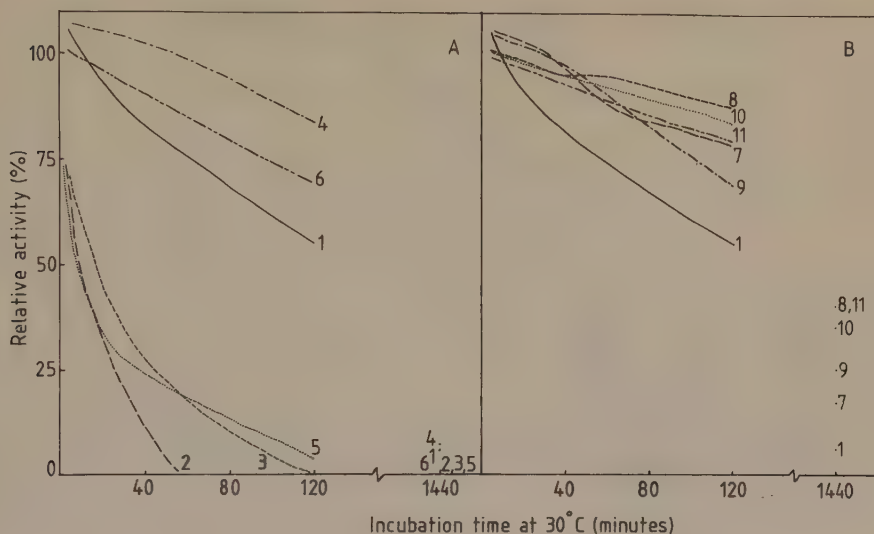


FIG. 1. The esterase activity of trypsin as a function of incubation time at 30°C (pH 9.0). The activity for each trypsin solution at zero time is used as standard value and is set to 100%.

A: pure trypsin (1); trypsin in SDS (2); trypsin in SHS (3); trypsin in KC_{12} (4); trypsin in KC_{16} (5); trypsin in Triton X-100 (6).

B: pure trypsin (1); trypsin in DPC (7); trypsin in HPC (8); trypsin in DAC (9); trypsin in HAC (10); trypsin in HTAB (11). The amphiphile to protein weight ratio was 0.40 g/g in all experiments.

The cationic amphiphiles all increase trypsin stability. Most effect is achieved by the hexadecyl compounds. Trypsin in the presence of HPC, HAC and HTAB possesses between 80 and 90% of the initial activity after 2 h. After 24 h about 40% of the initial value is still obtained.

To establish the mechanism of deactivation, trypsin samples incubated at 30°C, were analysed for cleavage products by SDS-urea PAGE. The electrophoretic pattern of trypsin in SDS showed a rapid decrease of the macromolecular band (MW 24000) while bands positioned in the molecular weight region 7000-3000 appeared. Samples of trypsin-SDS incubated for 2 h only have traces of the 24000 MW band. Pure trypsin and trypsin in HPC also show a decreased amount of the intact protein and an increased amount of low molecular weight peptides as a function of incubation time. The hydrolysis rate differs though: pure trypsin incubated for 24 h, resembles trypsin in

SDS incubated for 2 h, while samples of trypsin in HPC still after 24 h have a distinct band in the macromolecular position.

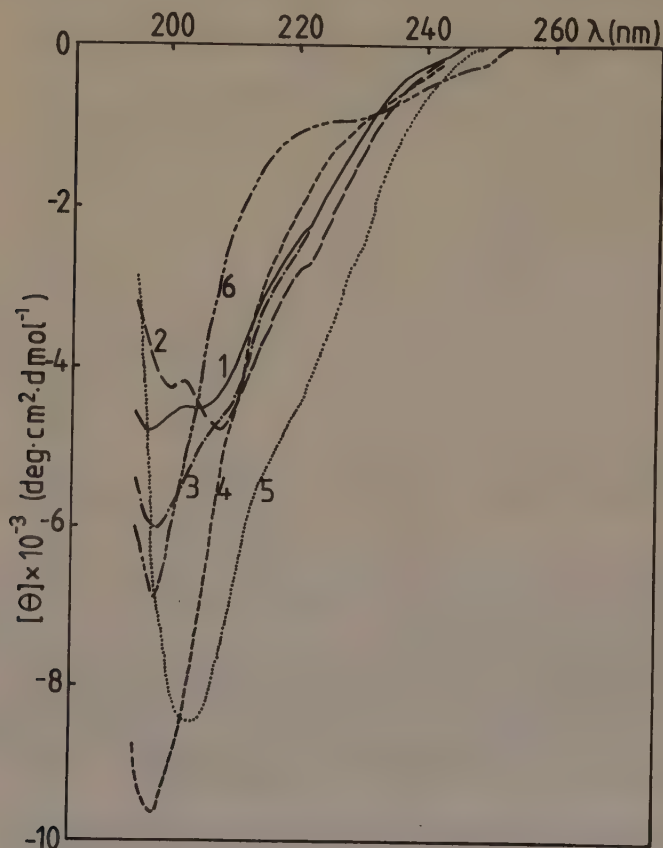


Fig. 2. The far UV circular dichroism spectra of trypsin at pH 9.0 with and without added amphiphiles. Curve 1 (—): pure trypsin, curve 2 (— —): trypsin in HPC, curve 3 (— · —): trypsin in KC_{12} , curve 4 (— · · —): trypsin in KC_{16} , curve 5 (·····): trypsin in SHS, curve 6 (— · — · —): trypsin in SDS. The amphiphile-to-trypsin weight ratio was 0.40 g/g in all experiments.

Circular Dichroism

CD-spectra in the far UV-region, yielding information about the backbone conformation of proteins, are shown in Fig. 2. Trypsin at pH 9.0 without additives (curve 1) gives a spectrum typical of a β -structure protein with low content of α -helical structure (12,13). HPC seems to increase the amount of ordered structure of trypsin (curve 2), indicated by the strengthening of the shoulder at 218-222 nm and the distinct minimum at 208 nm. Furthermore, the minimum at 196-198 nm seen in the trypsin spec-

trum, typical of unordered structure, is diminished. KC_{12} (curve 3), however, seems to intensify the band at 196-198 nm, whereas the CD-signal around 220 nm is less effected by this amphiphile. The other anionic amphiphiles have a much more drastic influence on the CD-spectrum of trypsin. KC_{16} (curve 4) as well as SDS (curve 6) give CD-spectra typical of flexible, fully disordered proteins (or protein segments), with weak dichroism above 220 nm and a strong negative peak at 198 nm. SHS, on the other hand, seems to reorganize the conformation of trypsin (curve 5). The CD-signal is strengthened in the complete wave-length region examined, showing a strong minimum centered around 204 nm.

The near-UV CD-spectra (not shown) confirm the surfactant-induced conformational changes of trypsin obtained in the far UV-region: a minor increase in intensity is obtained at addition of cationic amphiphiles, a small decrease in intensity at addition of KC_{12} , and spectra reflecting a more thorough disruption of the tertiary structure at addition of SDS, SHS and KC_{16} .

DISCUSSION

The anionic amphiphiles SDS, SHS and KC_{16} all retard the esterolytic activity of trypsin. This effect is consistent with a major disruption of the protein conformation as seen by the CD-measurements. The hydrocarbon sulphates with twelve or more carbon atoms are known to be strong denaturation agents (14). Trypsin in acidic solution, has been reported to bind SDS in a cooperative process with simultaneous gross unfolding (15). Denaturing effects on proteins by long-chain fatty acids have also been reported earlier (16).

The circular dichroism spectra of trypsin in the presence of anionic amphiphiles reported in this investigation exhibit interesting differences. Hexadecanoate induces a spectrum typical of an unfolded globular protein. This spectrum is, both in band position and intensity, very close to the one obtained on thermally destabilized trypsin without additive. SDS causes a CD-pattern similar to the one with KC_{16} , but with lower intensity. The CD-spectrum of trypsin-SDS is, however, contradictory to the ones reported by Jirgensons (17) on trypsin with added decyl and dodecylsulphates at pH 3. These show an extensive reorganization of trypsin conformation, yielding CD-spectra typical of a protein with high α -helical content. Trypsin is inactivated at acidic pH-values, and this pH induced transition involves a decrease in ordered structure (18). This transition, combined with the

effect of electrostatic forces, probably influence the mode of interaction. Hamed et al. (19) have shown that the α -helical content of proteins in presence of SDS is enhanced at acidification, due to titration of side-chain carboxyl groups. Hexadecylsulphate, however, seems to refold trypsin at the conditions used in the present investigation. The CD spectrum suggests formation of β -structure, and, as indicated by the negative peak at 204 nm, probably also a considerable amount of nonperiodic, but constrained, folding. This negative peak is also seen in the spectrum of native trypsin, but with a much lower intensity. Obviously, the interaction between trypsin and alkyl sulphates is sensitive to both pH and chain-length, emphasizing the complexity in protein-surfactant interactions.

It is well established that the binding of SDS to globular proteins, accompanied by a major change of the protein conformation, is a co-operative process. The unfolded protein state (D) is considered to be stable only if the binding sites on the unfolded molecule are saturated or nearly saturated (14,1,2). The two-state process might be described by the equilibrium: $N + n \text{ SDS} \rightleftharpoons \text{D}(\text{SDS})_n$, where N denotes the native protein. A distribution between folded and unfolded protein molecules can thus be assumed at SDS concentrations in the transition range. The decrease in trypsin activity achieved by SDS, SHS and KC_{16} can therefore be interpreted as a deactivation of a fraction of the trypsin molecules.

The two-state mechanism for protein unfolding by SDS also offers an explanation for the enhanced deactivation of trypsin at 30°C. The deactivation is accompanied by an increased hydrolysis rate, according to the SDS-PAGE pattern. Two simultaneously occurring processes might be assumed: (1) an increased binding of SDS to the protein at elevated temperature, shifting the equilibrium towards the unfolded state, and (2) an increased proteolytic rate as a result of elevated temperature and increased susceptibility of the substrate (i.e. unfolded trypsin molecules). Digestion of trypsin-SDS complexes makes a redistribution of SDS possible, thereby unfolding new trypsin molecules etc. The stability and conformation results indicate that hexadecanoate acts similar to SDS.

Dodecanoate is distinguished from the other anionic amphiphiles examined by its ability to increase esterolytic activity and stability of trypsin. In contrast to the activity measurements, circular dichroism shows an increase in disordered structure of trypsin by addition of this amphiphile. Since activity is remained, this indicate a perturbation of the structure, which might be of the same type as the gradual pH-induced

transition seen at acidification of pure trypsin. In combination with strengthened electrostatic repulsion forces, this can account for the slow-down of the self-digestion. In accordance with our results, Imoto et al. (16) have reported an increased protein unfolding by soaps when the chain length increases from dodecyl to hexadecyl.

Neither the nonionic nor the cationic surfactants used in this investigation deactivate trypsin. On the contrary, the cationic ones increase both esterolytic activity and stability of trypsin. An explanation for these observations might be found in the circular dichroism spectrum of trypsin in HPC, which indicates a more ordered structure compared to pure trypsin. A similar effect on trypsin, i.e. an increased activity and stability (11) in connection with an increased ordered structure (18,20) has been registered by addition of Ca^{2+} . The increased resistance towards autohydrolysis achieved by Ca^{2+} as well as by the cationic amphiphiles can thus be explained by the decreased susceptibility of the macromolecular substrate (i.e. trypsin) to tryptic digestion. A contribution to this effect might be derived from a strengthening of electrostatic repulsion forces.

In spite of the low affinity reported for nonionic surfactants to globular proteins (7) Triton X-100 increases the stability of trypsin at 30°C. No effect is, however, seen on the esterolytic activity against the low molecular weight substrate BAEE. The slow-down in the hydrolysis of trypsin might arise from steric hindrance towards a macromolecular substrate.

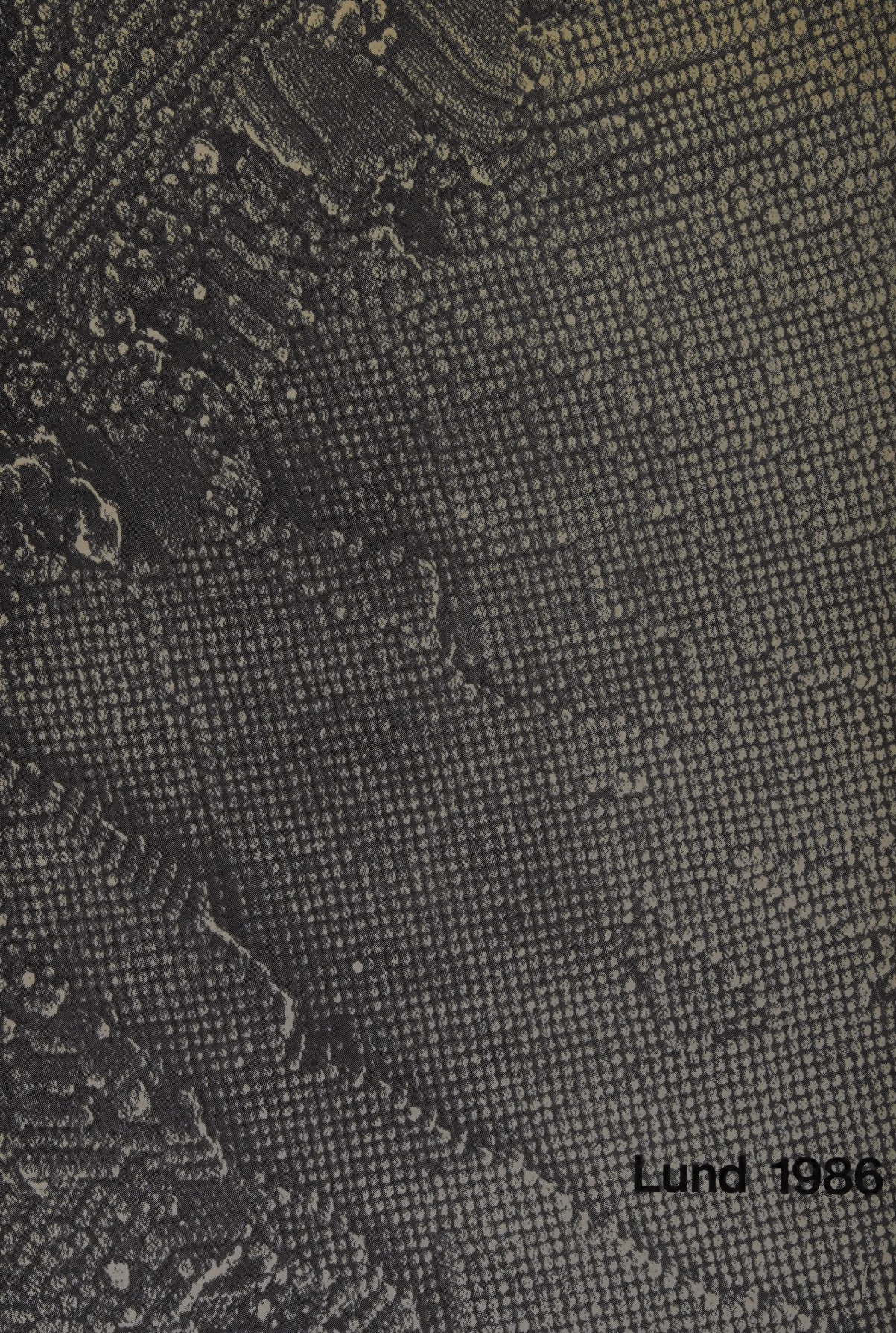
The obtained effects of the amphiphiles on trypsin suggest the possibility of regulating trypsin activity and autohydrolysis by selecting proper amphiphile and surrounding conditions. Depending on the amphiphile chosen, it is possible to get an unfolding/refolding of the protein structure, thereby causing loss of enzymatic activity. The unfolding can increase the susceptibility of trypsin as a substrate. It is also possible to get an increase in activity combined with improved stability. The importance of the experimental conditions is reflected by the drastic difference in effect on trypsin conformation by SDS at different pH values.

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